Insights into the Mechanism of 3-Deoxy-D-arabino-heptulosonate 7-Phosphate Synthase (Phe) from Escherichia coli Using a Transient Kinetic Analysis*

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Escherichia coli phenylalanine-sensitive 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase (DAHP synthase) catalyzes the net aldol condensation of phosphoenolpyruvate and erythrose-4-phosphate to form 3-deoxy-D-arabino-heptulosonate 7-phosphate and inorganic phosphate. For the first time, the presteady-state kinetic analysis of the Phe-sensitive DAHP synthase from E. coli is reported. The steady-state and presteady-state kinetic parameters of the DAHP synthase reconstituted with Mn(II), Cu(II), and Zn(II) were compared. These studies showed the following: 1) product release is rate-limiting for all of the three metal ions studied under physiologically relevant conditions; 2) concentration of the active sites of the metal-containing DAHP synthase is increasing from Mn- (30%) to Zn- (52%) and to Cu-DAHP synthase (88%); 3) rate constant for product formation is higher in Mn- (130–200 s⁻¹) than Cu- (55 s⁻¹) and Zn-DAHP synthase (6.8 s⁻¹); and 4) steady-state rate constant, product release is higher for the Mn- (70 s⁻¹) than for Cu- (5.6 s⁻¹) and Zn-DAHP synthase (1.8 s⁻¹). In addition, an examination of the reaction kinetics at lower pH reveals that for Cu-DAHP synthase, product release is no longer rate-limiting, whereas the Mn- and Zn-DAHP synthase show a slower rate of product formation, suggesting that the intermediate formation becomes rate-limiting in product formation. Also, a deuterium-isotope effect on the burst rate constant of product formation for Mn-DAHP synthase was observed at pH 6.0. This supports the hypothesis that the role of metal ion in E. coli DAHP synthase is to position the amino acids with the appropriate geometry required to coordinate and activate the water molecule.

The first committed step in the biosynthesis of shikimic acid, which ultimately leads to the synthesis of aromatic amino acids, is catalyzed by 3-deoxy-arabino-heptulosonate 7-phosphate (DAHP) synthase. The biosynthetic pathway of aromatic amino acids is absent in mammals, rendering the enzyme an attractive molecular target for antibiotic design. A putative inhibitor for the enzyme would inhibit bacterial growth while leaving the host organism unaffected.

Over the years, DAHP synthase has been isolated from a wide range of organisms ranging from bacteria to eukaryotes (1–5). Escherichia coli harbors three DAHP synthase isoforms, each subject to feedback inhibition by one of the three aromatic amino acids (tyrosine, phenylalanine, and tryptophan, respectively). In the present study, we have focused on the phenylalanine-sensitive DAHP synthase encoded by E. coli aroG gene. The enzyme catalyzes the net aldol condensation of phosphoenolpyruvate (PEP) and erythrose 4-phosphate (E4P) to form DAHP and inorganic phosphate (Pi) (Scheme 1).

The degree of sequence homology between the E. coli DAHP synthase and the 3-deoxy-D-manno-2-octulosonic acid-8-phosphate (KDO8P) synthase and the conserved active site residues between the two enzymes allow us to extrapolate the mechanistic findings from one system to the other (6). Previous experiments on KDO8P synthase showed the reaction is irreversible (7) and suggest the presence of an acyclic hemiketal phosphate intermediate in the catalytic mechanism of this enzyme (8, 9). Also, experiments using radiolabeled H₂¹⁸O established that the reaction involves the nucleophilic attack of a water molecule on C-2 of PEP, followed by or in concert with the nucleophilic attack of C-3 of PEP on the C-1 of arabinose 5-phosphate. A similar mechanism can be envisioned for the E. coli DAHP synthase. Indeed, the most recent crystal structure (10) revealed the presence of seven water molecules in the active site of the enzyme, with two of them positioned in close proximity to the C-2 of PEP that may serve as the nucleophile in the reaction (Scheme 1).

Unlike KDO8P synthase, which can catalyze this reaction either in a metal-dependent or metal-independent fashion, the DAHP synthases isolated thus far are all metal-dependent. From the first time DAHP synthase was isolated from E. coli in 1969 (2), there has been an ongoing controversy in the literature regarding the nature of the metal ion at the active site of this enzyme. A variety of metal ions have been proposed to reside at the active site of DAHP synthase under physiological conditions. They include cobalt (2, 11), iron (4, 12), or copper (13). Today, after more than 30 years of research, the nature of the physiological metal ion at the active site of DAHP synthase remains an open question.

A major objective of this work is to decipher the role of metal ion at the active site of DAHP synthase and to gain insights into the mechanism of product formation by determining the steady-state and presteady-state kinetic parameters of the enzyme.
zyme reconstituted with different metal ions. Potential metal coordinating residues, Cys-61 and His-268, have been shown to play an important role in the catalytic activity of DAHP synthase (14, 15). Mn(II) (hard metal) and Cu(II) or Zn(II) (softer metals as compared with Mn(II)) coordinate different types of ligands, with Mn(II) preferring oxygen ligands and Cu(II) or Zn(II) preferring nitrogen and thiol ligands (16). Accordingly, a detailed kinetic analysis of the enzyme reconstituted with Mn(II), Cu(II), and Zn(II) has been performed.

The parameters derived from steady-state and presteady-state burst experiments for the Mn-, Cu-, and Zn-DAHP synthase were compared to address the following questions. (i) What is the rate-limiting step in the kinetic mechanism of Mn-, Cu-, and Zn-DAHP synthase? (ii) What is the concentration of the active sites for the three forms of DAHP synthase? (iii) What are the relative rate constants of chemical catalysis for product formation? (iv) Which step in the catalytic mechanism is affected by lowering the pH? (v) What may the steady-state and presteady-state kinetic parameters reveal regarding the ability of the metal ion to facilitate the reaction? In addition, the deuterium kinetic isotope effect on the rate constant of product formation was determined to gain information on the metal ion composition in the enzyme, prior to all kinetic experiments, the enzyme was treated with EDTA to remove the metal ions bound to the isolated enzyme. The enzyme was dialyzed overnight against 50 mM Tris, pH 7.5, containing 10 mM EDTA followed by successive dialysis (two times for 4 h) against the same buffer but in the absence of EDTA. All buffers were treated with Chelex-100. The glassware and the spectrophotometric cuvettes were washed with 2 M KOH. After dialysis, the enzyme was concentrated using a Centriprep YM-10 (Millipore), diluted to the desired concentration prior to the rapid chemical quench experiments.

**Steady-state Kinetic Analysis; Mn(II), Cu(II), and Zn(II) Dependence of the Steady-state Rate—**The reactions were performed at 25 °C in 1 ml of 50 mM Tris, pH 7.5. The reactants were added in the following order: the metal ions (0–500 μM) and the apoDAHP synthase (50 nM) were preincubated for 10 min, then 300 μM PEP was added to the reaction mixture, and the reaction was initiated by the addition of the second substrate, E4P. The consumption of PEP was monitored spectrophotometrically. The parameters derived from steady-state and presteady-state kinetic analysis of a DAHP synthase and provides insight into these questions that may offer valuable input for the design of novel enzyme inhibitors.

**EXPERIMENTAL PROCEDURES**

In this section and throughout the paper we used DAHP synthase to refer to the isolated “as is” DAHP synthase and apoDAHP synthase for the EDTA-treated DAHP synthase (metal-free).

**Materials—**PEP, E4P, manganese chloride (MnCl₂), copper chloride (CuCl₂), magnesium chloride (MgCl₂), ATP, potassium monophosphate (KH₂PO₄), ammonium chloride (NH₄Cl), inorganic pyrophosphatase, triethylamine, and Chelex-100 were purchased from Sigma. [1-14C]Pyruvate was obtained from American Radiolabeled Chemicals, and the high pressure liquid chromatography MonoQ 5/5 anion-exchange column was from Amersham Biosciences.

**Purification of DAHP Synthase—**DAHP synthase was purified as reported previously (17). Protein concentration was determined by using an extinction coefficient for DAHP synthase at 280 nm of 40.5 mM⁻¹ cm⁻¹ (18). The concentration of the active sites was determined as described below, and the catalytic rate was calculated based on concentration of the active sites determined from burst experiments.

**[1-14C]PEP Synthesis—**Radiolabeled PEP was enzymatically synthesized from [1-14C]pyruvate by coupling the pyruvate phosphate dikinase reaction to inorganic pyrophosphatase reaction. Pyruvate phosphokinase was a generous gift from Prof. Dunaway-Mariano at the University of New Mexico. The radiolabeled PEP was purified by Q-Sepharose anion-exchange chromatography using a linear gradient of 20 mM to 1 M triethylamine bicarbonate (TEAB). The fractions containing PEP were identified by absorbance at 232 nm. Final stock solutions (5.6 mM) contained 29,300 dpm/mmol.

**Metal Ion Removal from DAHP Synthase and Reconstitution with Mn(II), Cu(II), or Zn(II) Metal Ions—**In order to eliminate heterogeneity in metal ion composition in the enzyme, prior to all kinetic experiments, the enzyme was treated with EDTA to remove the metal ions bound to the isolated enzyme. The enzyme was dialyzed overnight against 50 mM Tris, pH 7.5, containing 10 mM EDTA followed by successive dialysis (two times for 4 h) against the same buffer but in the absence of EDTA. All buffers were treated with Chelex-100. The glassware and the spectrophotometric cuvettes were washed with 2 M KOH. After dialysis, the enzyme was concentrated using a Centriprep YM-10 (Millipore), diluted to the desired concentration prior to the rapid chemical quench experiment and reconstituted with 2 mM MnCl₂ or 2 mM CuCl₂. For the reconstitution with ZnCl₂, the enzyme was first diluted to 100 nM. An equimolar concentration of ZnCl₂ was then added, and the reconstituted enzyme was concentrated to the desired concentration.

**Path B**

**SCHEME 1. Proposed reaction mechanisms for DAHP synthase.** Path A, stepwise mechanism for the formation of a linear hemiketal bisphosphate intermediate. Path B, the hemiketal intermediate formation takes place through an oxocarbonium intermediate (concerted mechanism).
metabolically by following the decrease of absorbance at 232 nm. The steady-state rate was calculated by using an extinction coefficient for PEP at 232 nm of 2.84 mM·cm⁻¹. The effect on the rate by metal ions was determined by comparing the rates with CuCl₂, ZnCl₂, and MnCl₂. There was no change in the kinetic behavior such as a lag in the reaction kinetics when the order of addition of reactants was changed.

To test the optimum amount of metal ion required to fully activate the enzyme, apoDAHP synthase (50-μl aliquots of 54 μM) was incubated with different MnCl₂ concentrations in 50 mM Tris, pH 7.5. After 10 min of incubation, 1 μl was removed and added to a 1-ml reaction mixture containing 1 mM E4P, 300 μM PEP in 50 mM Tris, pH 7.5. No additional metal ion was added to the reaction mixture. The consumption of PEP was monitored spectrophotometrically by following the decrease of absorbance at 232 nm. The steady-state rate was calculated by using an extinction coefficient for PEP at 232 nm of 2.84 mM·cm⁻¹. The effect on the rate by metal ions was determined by comparing the rates with CuCl₂, ZnCl₂, and MnCl₂. There was no change in the kinetic behavior such as a lag in the reaction kinetics when the order of addition of reactants was changed.

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Transient Kinetic Analysis of E. coli DAHP Synthase (Phe)

**RESULTS**

When isolated from *E. coli*, recombinant DAHP synthase is heterogeneous in terms of metal ion content with 30% of DAHP synthase containing Fe(II) and 10% containing Zn(II), Cu(II), and other metal ions, whereas most of the enzyme (>60%) does not seem to have any metal ion bound at the active site. Reconstitution of apoDAHP synthase with different metal ions showed that a wide range of metal ions activate the enzyme. Although the highest catalytic rate is achieved with Mn(II), Zn(II) is the metal ion with the highest affinity for DAHP synthase (19). Considering the different chemical properties of the two metal ions (16), one of the objectives was to investigate how these differences are reflected on the kinetic landscape of the reaction catalyzed by Mn-DAHP synthase versus Zn-DAHP synthase. The chemical properties of Cu(II) (type of ligands and coordination geometry) are similar to those of Zn(II), but the magnetic (EPR-active) and optical properties of Cu(II) could be further exploited in the kinetic analysis of DAHP synthase.

In the first part of the "Results" section, the steady-state and presteady-state kinetics of the Mn-, Cu-, and Zn-DAHP synthase are presented, whereas the second part focuses on the pH dependence and deuterium kinetic isotope effect of the presteady-state kinetic parameters of Mn-, Cu-, and Zn-DAHP synthase. The main goals of these experiments are as follows: 1) determine the correlation between metal ion affinity and the concentration of the active sites, 2) determine the rate-limiting step in the catalytic cycle, and 3) gain insight into the steps that lead to product formation. The main hypothesis for this work was that a comparison of the presteady-state kinetic parameters for Mn-, Cu-, and Zn-DAHP synthase at different pH values along with the investigation of the deuterium isotope effect on the kinetics of product formation would provide insight into the role of metal ion at the active site of DAHP synthase.

**Mn-DAHP Synthase**

The Dependence of *k*ₘₐₓ on MnCl₂ Concentration—Manganese has been reported as the metal ion that gives the highest steady-state rate (19). In the present study, the *Kₘ* (1 μM) and *k*ₘₐₓ (23 s⁻¹) values were determined by following the consumption of PEP in a continuous spectrophotometric assay (Fig. 1). The *k*ₘₐₓ value was calculated based on protein concentration by assuming a concentration of the active sites equal to protein.
concentration. However, the results of burst experiments with the Mn-DAHP synthase (see below and Fig. 2) show that only 30% of the enzyme is active. Therefore, the corrected \( k_{\text{cat}} \) value based upon the concentration of the active sites is 75 s\(^{-1}\) (Fig. 1). This value is in accordance with the previously reported rate determined for the enzyme (19).

**Determining the Concentration of the Active Sites for the Mn-DAHP Synthase**—A burst experiment was performed to determine the concentration of the active sites. The data presented in Fig. 2 (solid circle) were fitted to Equation 1, and the resulting parameters are as follows: a burst rate of 200 s\(^{-1}\), amplitude corresponding to 28% of the protein concentration, and steady-state rate of 36 s\(^{-1}\) (Table I). There is a variance between the \( k_{\text{cat}} \) determined in the steady-state assay (75 s\(^{-1}\)) and the \( k_{\text{cat}} \) determined in the presteady-state assay (36 s\(^{-1}\)). A comparison of the steady-state and pre-steady-state experiments for Mn-DAHP synthase (\( K_{\text{Mn}}^\text{cat} \) 1 mM) and Cu-DAHP synthase (\( K_{\text{Cu}}^\text{cat} \) 120 nM, see below) reveals that whereas 2 mM Mn(II) inhibitory for the steady-state rate, 2 mM Cu(II) is not, despite the almost 10-fold lower \( K_d \) of Cu(II). Therefore, in the case of Mn-DAHP synthase, we considered it necessary to look at the effect of the metal ion to enzyme ratio on the steady-state rate to explain the apparent discrepancy between the steady-state rate obtained from the pre-steady-state burst experiment and the rate obtained from the steady-state experiments. Most surprisingly, it was found that only in a relatively narrow range of Mn(II)/DAHP synthase concentration ratios (80–100) did the enzyme achieve maximum specific activity. Higher Mn(II) concentrations resulted in a rapid drop in the enzyme specific activity. This is consistent with the pre-steady-state burst experiment in which a ratio of Mn(II)/DAHP synthase of 150 was used. The expected steady-state rate at this ratio of Mn(II)/DAHP synthase is 35 s\(^{-1}\), which is in good agreement with the steady-state rate determined from the burst experiment.

**Cu-DAHP Synthase**

The Dependence of \( k_{\text{cat}} \) on CuCl\(_2\) Concentration—The dependence of DAHP synthase rate upon CuCl\(_2\) concentration was determined. The \( k_{\text{cat}} \) was 5 s\(^{-1}\) and the \( K_d \) for Cu(II) was 120 nM (Fig. 3). The values are based on protein concentration. Based on the results from the burst experiments described below, it was determined that 88% of the protein active sites were active adjusting the \( k_{\text{cat}} \) to 5.6 s\(^{-1}\).

**Determining the Concentration of the Active Sites for the Cu-DAHP Synthase**—To confirm the catalytic rate determined in the previous section and to gain information on the rate of product formation, a burst experiment for the Cu-DAHP synthase was performed. Similar to Mn-DAHP synthase, product release was also rate-limiting for Cu-DAHP synthase. The burst rate was 55 s\(^{-1}\); the amplitude corresponded to 88% of protein concentration; and the steady-state rate was 5.6 s\(^{-1}\) (Table I and Fig. 4, solid circles). The rates determined in the steady-state experiments were corrected by the concentration of the active sites, and the data were replotted (Fig. 3, open circles).

**Zn-DAHP Synthase**

**Steady-state Rate Dependence on ZnCl\(_2\) Concentration**—ApodAHPSynthasedeterminedthroughreconstitutionwithMnCl\(_2\)andCuCl\(_2\). The steady-state rate was the same over the range of metal ion concentrations used (3 s\(^{-1}\)). Lowering the ZnCl\(_2\) concentration below the enzyme concentration resulted in a decrease in the steady-state rate most likely because of a lower metal ion to enzyme stoichiometry.

**Determining the Concentration of the Active Sites for the Zn-DAHP Synthase**—ApodAHPSynthasedeterminedthroughreconstitutionwithMnCl\(_2\)andCuCl\(_2\). The steady-state rate was the same over the range of metal ion concentrations used (3 s\(^{-1}\)). Lowering the ZnCl\(_2\) concentration below the enzyme concentration resulted in a decrease in the steady-state rate most likely because of a lower metal ion to enzyme stoichiometry.

**The Effect of pH on the Chemistry of DAHP Synthesis**—To investigate the effect of pH on the rate of chemistry, pre-steady-state burst experiments were performed at lower pH values, and the experimental results for Mn-, Cu-, and Zn-DAHP synthase are summarized in Table I. Most surprisingly, the nature of metal ion seemed to have a major effect on the pH dependence of DAHP synthase. Zn-DAHP synthase was the least affected by the change in pH. The concentration of the active sites (52%), the burst rate (6.8–7.4 s\(^{-1}\)), and the steady-state rate (1.7–1.8 s\(^{-1}\)) were basically identical at the two pH values (Fig. 6). In the case of Mn-DAHP synthase, although the concentration of the active sites remained the same (28%), the burst (45 s\(^{-1}\) at pH 6.0) and the steady-state rates (24 s\(^{-1}\) at pH 6.0) were significantly affected by the change in pH (Fig. 2, open circle). The Cu-DAHP synthase showed the most drastic effect. At pH 6.5, the chemistry leading to product formation became rate-limiting, and the rate of product formation was 22 \( \mu \)mol/s, which translates into a rate of 2.3 s\(^{-1}\) (Fig. 4, open circle).

**Deuterium Isotope Effect on Mn-DAHP Synthase Reaction**—To investigate the presence or absence of a deuterium kinetic isotope effect on the product formation, two sets of burst experiments were performed at pH 6.0 and at pH 6.8 in protinated and deuterated 50 mM BTP buffer for Mn-DAHP synthase. A kinetic isotope effect on the rate constant of product formation would suggest that the steps leading to intermediate formation become rate-limiting for product formation. Performing the reaction at the physiologically relevant pH 6.8 or 7.5.

![Fig. 2. Burst experiments for Mn-DAHP synthase, 8.4 \( \mu \)M apo-DAHP synthase, 2 mM MnCl\(_2\), and 25.2 \( \mu \)M [\(^{14}\)C]PEP were reacted with 500 \( \mu \)M E4P. The reaction was performed in 50 mM BTP pH 7.6 (●) and 50 mM BTP pH 6.0 (□). The parameters determined by fitting the data to a burst equation were 28% active sites, rate constant for product formation of 200 and 45 s\(^{-1}\), and steady-state rate of 36 and 24 s\(^{-1}\), respectively.](http://www.jbc.org/xx)
resulted in burst rate constants higher than 200 s$^{-1}$, precluding a reliable estimation of the kinetic isotope effect under these conditions. However, an isotope effect of 2 was observed on the rate constant of product release (Fig. 7, panel A). Lowering the reaction temperature did not lower the rate constant of product formation to values below 100 s$^{-1}$ (data not shown). On the other hand, at pH 6.0 a deuterium kinetic isotope effect of 2 was observed for the burst rate constant (the rate constant of product formation) (62 versus 34 s$^{-1}$), whereas no significant isotope effect was observed on the rate of product release (24 versus 18 s$^{-1}$) (Fig. 7, panel B). To confirm further the rate constant of product formation, a pre-steady-state single turnover experiment was performed at pH 6.0 in protonated and deuterated E4P. The reaction was performed in 50 mM Tris, pH 7.5, under saturating concentrations of E4P (1 mM) and PEP (300 μM). The metal ion concentration (CuCl$_2$) was varied between 0 and 100 μM. ApoDAHP synthase was added to a final concentration of 50 nM. The steady-state rate of Cu-DAHP synthase based on protein concentration (●) (4.8 s$^{-1}$, $K_D = 120$ nM) and on concentration of the active sites (○) (5.6 s$^{-1}$, $K_D = 120$ nM) is plotted versus CuCl$_2$ concentration.

**TABLE I**

Summary of pre-steady-state kinetic parameters

|            | Mn-DAHPS |            | Zn-DAHPS |            | Cu-DAHPS |
|------------|----------|------------|----------|------------|----------|
| pH 6.0     |          |            |          |            |          |
| % active sites | % active sites | % active sites | % active sites | % active sites | % active sites |
| Burst rate s$^{-1}$ | Burst rate s$^{-1}$ | Burst rate s$^{-1}$ | Burst rate s$^{-1}$ | Burst rate s$^{-1}$ | Burst rate s$^{-1}$ |
| 28 45 24 | 52 7.4 1.7 | 88 55 2.3 |
| pH 7.6     |          |            |          |            |          |
| % active sites | % active sites | % active sites | % active sites | % active sites | % active sites |
| Burst rate s$^{-1}$ | Burst rate s$^{-1}$ | Burst rate s$^{-1}$ | Burst rate s$^{-1}$ | Burst rate s$^{-1}$ | Burst rate s$^{-1}$ |
| 28 200 36 | 52 6.8 1.8 | 88 55 2.3 |

* pH 6.5 for the Cu-DAHPS.
steady-state rates of 24 and 18 s −1 for Mn(II), with one of the ligands being the thiol group of Cys-61. Cysteine is a very unlikely ligand for Mn(II) because none of the manganese-containing enzymes currently present in the Protein Data Bank contain a cysteine coordinated to Mn(II). On the other hand, there are numerous examples of cysteine ligated to Zn(II) or Cu(II). Although there is no available crystal structure of the Cu- or Zn-DAHP synthase, there is evidence that Cys-61 is essential for the enzyme activity (15, 18, 23). It remains to be established whether Cys-61 is directly involved in coordinating the Cu(II) or Zn(II), as suggested by mutational and UV-visible spectroscopic studies (18, 23), or whether its role is in substrate binding and secondary shell interactions (15).

The results of our steady-state and presteady-state experiments show that although DAHP synthase has higher specific activity with Mn(II), only 30% of the enzyme catalytic sites are active. On the other hand, the reconstitution of DAHP synthase with Cu(II) results in almost 100% concentration of the active sites. These results correlate with the relative affinity of DAHP synthase for these metal ions, with Cu(II) binding tighter to the enzyme than Mn(II). On the basis of these results, one would expect a high concentration of the active sites for Zn-DAHP synthase, with Zn(II) having the highest kcat/KM values from all the metal ions tested. Surprisingly, reconstitution of DAHP synthase with Zn(II) led to unexpected results. Upon performing the reaction at different enzyme concentrations, it was found that although the percent of concentration of the active sites and the steady-state rate were constant (52% and 1.7 s −1), the rate constant of product formation was decreased as the enzyme concentration was increased. By extrapolating these rates at the enzyme concentrations used in the steady-state assay (100 nM), a burst rate constant between 30 and 60 s −1 is expected. Interestingly, lowering the Zn(II) concentration in the steady-state assay below the enzyme concentration resulted in a decrease in the steady-state rate. These results suggest that in a more diluted state, the reconstitution with Zn(II) results in close to 100% concentration of the active sites. The steady-state rate of 3.2 s −1 determined under steady-state conditions (100% active sites) is double the steady-state rate determined by the burst experiments (1.7 s −1, 52% concentration of the active sites). These kinetic parameters are closer to those for Cu-DAHP synthase, suggesting similar catalytic properties for the two forms of enzyme. Although one would expect the difference between the Mn-DAHP synthase and Cu- or Zn-DAHP synthase to be mainly in the rate of chemistry (e.g. product formation), smaller or little difference might be expected in the rate of product release unless the metal ion is involved in coordinating the product or is indirectly involved in product release. The steady-state rate corresponding to the rate of product release is increasing from Zn-DAHP synthase to Cu-DAHP synthase and to Mn-DAHP synthase (Table I). With the exception of Cu-DAHP synthase, this parallels the Irving-Williams series of stability constants for metal complexes (24). Cu-DAHP synthase deviation from the Irving-Williams series is not unprecedented in biological systems (25). The coordination geometry of a certain metal within the protein environment is likely to deviate from the standard coordination geometries known to occur in solution. In addition, for copper one must consider the Jahn-Teller effect that leads to deviations from the characteristic square planar coordination geometry.

The results reflect the importance of metal ion at the active site of DAHP synthase. The concentration of the active sites, the rate constant of product formation, and the rate of product release are dependent on the nature of the metal ion bound to the enzyme. A question arising from these experiments targets the relative importance of the concentration of the active sites versus the rate constant of product formation in biological
systems. For the enzymes involved in biosynthetic pathways, where the enzymes form multiprotein complexes and the product of one enzymatic reaction is handed over to the next enzyme in the pathway, the concentration of the active sites might serve as a main criterion in establishing the nature of the metal ion cofactor at the active site of the enzyme. For example, if only 30% of one of the enzymes in the pathway is active, the next enzyme in the pathway can only use 30% of its enzymatic sites even though the concentration of the active sites of this enzyme is 100%. Future experiments in which Mn- and Cu-DAHP synthase will be coupled to dehydroquinate synthase, the next enzyme in the chorismate biosynthetic pathway, will prove or disprove this hypothesis.

Several structural (10, 22) and kinetic (26) studies on DAHP synthase and KDO8P synthase led to the hypothesis that the initial step in the DAHP or KDO8P synthesis reaction is a nucleophilic attack of water on C-2 of PEP (Scheme 1, path A) rather than the formation of a transient oxocarbonium intermediate (Scheme 1, path B). Although the issue is still under debate, we must answer first the question about the role of the metal ion at the active site of these enzymes. Is the metal ion directly involved in activating the water molecule for the nucleophilic attack, or is its role more structural, arranging the active site residues in a position favorable for water activation? In the recently published crystal structure of DAHP synthase (10), two water molecules have been identified as being candidates for the potential nucleophile in the reaction. One of them is indeed coordinated by Mn(II), but the other one is not and could serve as the nucleophile.

The two main parameters that govern the nucleophilic character of the active water molecule are the pH of the solution and the $pK_a$ of the water molecule. Assuming that the water acting as a nucleophile in the initial steps of the DAHP synthase reaction is coordinated by the active site metal ion, the $pK_a$ of water will be dependent on the nature of metal ion and on the interaction with the second shell ligands. Based on the Lewis acidity character of the metal ion coordinating the water, in solution, the $pK_a$ of the water coordinated to copper (7.5) or zinc (9.6) is expected to be lower than the $pK_a$ of the water coordinated by manganese (11.5). Based on this concept, upon a change in pH from 6.0 to 7.5, one would expect a stronger pH dependence of the burst rate constant for Cu-DAHP synthase followed by Zn- and Mn-DAHP synthase. Indeed, the rate constant of product formation showed the strongest pH dependence for Cu-DAHP synthase. However, Mn-DAHP synthase showed stronger pH dependence than Zn-DAHP synthase, leading to the conclusion that the Lewis acidity character of the metal ion is not entirely responsible for modulating the $pK_a$ of the water molecule. The first clue for the role of metal ion at the active site of DAHP synthase was the presence of an isotope effect at pH 6.0. This along with the pH dependence data, which suggest a change in the rate-limiting step leading to product formation at lower pH, provide us with a first time look at the steps leading to product formation. Although these types of experiments do not distinguish between path A and path B in Scheme 1, they tell us that at lower pH the steps leading to intermediate formation become totally rate-limiting in the rate of product formation. As shown in Scheme 1, both path A and path B involve the nucleophilic attack of a hydroxide ion on C-2 of PEP. The experiments presented in this work show that at lower pH the nucleophilic character of this hydroxide ion is diminished, which results in a lower rate constant of intermediate formation. If the role of metal ion is to directly coordinate and activate the water molecule, the change in pH from 6.8 or 7.5 to 6.0 should not affect the rate constant of intermediate or product formation for Mn-DAHP synthase ($pK_a$ of 11.5) or Zn-DAHP synthase ($pK_a$ of 9.6), although it would affect the rate constant of product formation for Cu-DAHP synthase ($pK_a$ of 7.5). However, if the role of metal ion is to bring together the amino acids required to coordinate and activate the water molecule, a change in pH is expected to influence the nucleophilicity of the resulting hydroxide ion and hence the rate constant of intermediate formation.

The presence of a deuterium isotope effect on the burst rate for Mn-DAHP synthase at pH 6.0 suggests that the rate constant of the water attack at C-2 of PEP (through path A or path B in Scheme 1) is rate-limiting in the overall chemistry leading to product formation. If the concentration of HO$^-$ ions were the only limiting factor in the rate of chemistry, one would expect a 10-fold change in the rate constant with every 1-unit change in pH. Neither Mn- nor Zn-DAHP synthase showed such a drastic dependence on pH, also suggesting a nonrate-limiting nucleophilic attack by water at pH 6.8 or 7.5. However, when the burst experiment was performed for Cu-DAHP synthase at lower pH, no burst in product formation was observed. Under these conditions, the chemistry leading to product formation became rate-limiting, and the change in pH resulted in a 20-fold change in the rate of product formation. This is more than expected for a 1-unit change in pH. It has been shown before that upon a change in pH from pH 6.0 to pH 8.0, Cu-DAHP synthase undergoes a conformational change with a possible change in the coordination sphere from a nitrogen donor to a sulfur donor, Cys-61 being one of the potential candidates (27). It is possible that the loss of this ligand upon lowering the pH is responsible for the drastic effect observed on the rate of product formation for Cu-DAHP synthase.

In addition to the insight these experiments have provided into the reaction mechanism of DAHP synthase and the related KDO8P synthase, they have also led to two other important findings to the field of metalloenzymes. The current dogma concerning the identity of the metal ion at the active site of the enzymes is that the metal ion giving the highest rate is more likely to be the metal ion assisting the catalytic reaction. Although the rate of catalysis is important, our results suggest that the concentration of the active sites as determined by metal affinity and intracellular concentration of metal ions should also be considered as a criterion in establishing the nature of the metal ion under physiological conditions. An effective comparison of reactivity and affinity and the assessment of their relative importance must take into consideration the metabolic pathway where the specific enzyme belongs. Our studies raised and tried to answer an important question for the growing field of systems biology. When performing cell/pathway modeling, should one consider only enzyme reactivity as defined by its steady-state parameters or the actual rate of product formation and active site concentration as determined from presteady-state burst experiments? The second finding is related to the role of the metal ion at the active site of the enzymes. In most of the metalloenzymes characterized thus far whose mechanisms involve a nucleophilic attack by water, the role of metal ion is to directly coordinate the water molecule and activate it for the nucleophilic reaction. Our results suggest that in DAHP synthase, the metal ion plays a crucial role in both stages of catalysis, i.e. chemistry leading to product formation and product release. The data imply an indirect role of the metal ion in activating the water molecule toward a nucleophilic attack and suggest that the second water molecule at the active site of the enzyme, not coordinated by the metal ion, serves as a nucleophile in the reaction.

To summarize: 1) at physiological pH, the rate-limiting step for Mn-, Cu-, and Zn-DAHP synthase is product release; 2) reconstitution with Cu(II) or Zn(II) results in higher concen-
tration of the active sites than reconstitution with Mn(II) even though both the steady-state rate of product release and the presteady-state rate constant of product formation are higher for the Mn-DAHP synthase than for Cu- or Zn-DAHP synthase; and most importantly, 3) the metal ion at the active site of the enzyme plays a more structural role, orchestrating the arrangement of the active site residues in a position favorable for water activation. In this view, the coordination geometries of different metal ions are the determinant for the nucleophilicity of the resulting hydroxide ion.

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