Probing the Role of Metal Ions in the Catalysis of Helicobacter pylori 3-Deoxy-d-manno-octulosonate-8-phosphate Synthase Using a Transient Kinetic Analysis*

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Apurba Kumar Sau, Zhili Li, and Karen S. Anderson‡

From the Department of Pharmacology, Yale University School of Medicine, New Haven, Connecticut 06520

3-Deoxy-d-manno-2-octulosonate-8-phosphate (KDO8P) synthase catalyzes the net condensation of phosphoenolpyruvate and D-arabinose 5-phosphate to form KDO8P and inorganic phosphate (P_i). Two classes of KDO8P synthases have been identified. The Class I KDO8P synthases (e.g. Escherichia coli KDO8P synthase) catalyze the condensation reaction in a metal-independent fashion, whereas the Class II enzymes (e.g. Aquifex aeolicus) require metal ions for catalysis. Helicobacter pylori (H. pylori) KDO8P synthase, a Zn^{2+}-dependent metalloenzyme, has recently been found to be a Class II enzyme and has a high degree of clinical significance since it is an attractive molecular target for the design of novel antibiotic therapy. Although the presence of a divalent metal ion in Class II KDO8P synthases is essential for catalysis, there is a paucity of mechanistic information on the role of the metal ions and functional differences as compared with Class I enzymes. Using H. pylori KDO8P synthase as a prototypical Class II enzyme, a steady-state and transient kinetic approach was undertaken to understand the role of the metal ion in catalysis and define the kinetic reaction pathway. Metal reconstitution experiments examining the reaction kinetics using Zn^{2+}, Cd^{2+}, Cu^{2+}, Co^{2+}, Mn^{2+}, and Ni^{2+} yielded surprising results in that the Cd^{2+} enzyme has the greatest activity. Unlike Class-I KDO8P synthases, the Class II metallo-KDO8P synthases containing Zn^{2+}, Cd^{2+}, Cu^{2+}, and Co^{2+} show cooperativity. This study presents the first detailed kinetic characterization of a metal-dependent Class II KDO8P synthase and offers mechanistic insight for how the divalent metal ions modulate catalysis through effects on chemistry as well as quaternary protein structure.

3-Deoxy-d-manno-2-octulosonate-8-phosphate (KDO8P)^1 synthase catalyzes a net aldol condensation reaction between D-arabinose 5-phosphate (A5P) and phosphoenolpyruvate (PEP) to form an unusual eight-carbon sugar KDO8P and inorganic phosphate (P_i). This is a key enzymatic reaction that controls the carbon flow in the biosynthetic formation of 8-carbon sugar 3-deoxy-d-manno-2-octulosonate (KDO). The KDO is an important constituent of lipopolysaccharides found in most Gram-negative bacteria (1) and plays a crucial role in this assembly process (1, 2). Because KDO8P synthase is essential for Gram-negative bacteria and not present in mammalian systems, it represents an attractive molecular target for the design of new antibiotics (3, 4).

Only one other enzyme found in nature catalyzes a related type of enzymatic reaction. The enzyme 3-deoxy-d-arabino-2-heptulosonate-7-phosphate (DAHP) synthase catalyzes a similar condensation reaction between PEP and erythrose-4-phosphate, a sugar containing one less carbon, to form DAHP and inorganic phosphate (P_i). The formation of the DAHP is the first committed step in the biosynthesis of the intermediate compounds chorismate and prephenate, which are precursors to the aromatic amino acids (Phe, Tyr, and Trp), catechols, and p-aminobenzoic acid as well as a number of other highly important microbial compounds (5). Both enzymes, KDO8P synthase and DAHP synthase, catalyze the condensation reactions with the same stereo-facial selectivity with respect to the double bond of PEP and the aldehyde moiety of the monosaccharide substrate (6–9) as well as with cleavage of the C-O bond of PEP (8, 10–12) rather than the more common scission of the P-O bond. Previous studies on Escherichia coli KDO8P synthase suggest the presence of an acyclic hemiketal phosphate intermediate, I, as shown in Scheme 1 (13). Accordingly, the reaction involves the nucleophilic attack of water/hydroxide on C2 of PEP followed by or in concert with the nucleophilic attack of C3 PEP on the aldehyde carbon of A5P.

Although there is little or no homology at the level of the primary sequence, the three-dimensional structures of E. coli KDO8P synthase and DAHP synthase have been reported and provide evidence that these two enzymes are not only mechanistically but also structurally related. Nonetheless, these two enzymes have evolved to use two different strategies to promote catalysis (14, 15). The catalytic activity of DAHP synthase is dependent on the divalent transition metal ion such as Mn^{2+} or Zn^{2+}. The kdsa gene encoding KDO8P synthase has been identified in other bacteria (16–18) and plants (16), and based on the early observations on the E. coli KDO8P synthase (19), it has been found that the enzyme does not require divalent metal ion for catalysis.

Recently it was shown, however, that the KDO8P synthase from the hyperthermophilic bacterium Aquifex aeolicus and similar species such as Aquifex pyrophilus require divalent metal cofactor for catalysis (20, 21), implying that there may be two classes of KDO8P synthases, one metal-independent and one metal-dependent. Thus KDO8P synthase represents one of the first examples where orthologous enzymes differ based on their requirement of the metal cofactor. The x-ray crystal struc-
Metal Ions in Catalysis for Class II KDO8P Synthases

One of the putative metallo-KDO8P synthases is encoded by H. pylori, an important human pathogen that colonizes the gastric mucosa and can cause gastritis and cause gastric ulcers (25–28). H. pylori infection was linked to an increased risk of developing some forms of gastric cancer and was classified as a carcinogen (29, 30). H. pylori KDO8P synthase is reported to be a zinc-metalloenzyme (24). The presence of the Zn$^{2+}$ has been shown to be catalytically important. The enzyme was inactivated by the treatment with metal-chelating agents such as EDTA and PDA. Although a preliminary study of the effect of metal ions on H. pylori KDO8PS has been previously reported (25), a detailed mechanistic understanding of this enzyme or other Class II-KDO8PS synthases is lacking.

Using H. pylori KDO8P synthase as a prototypical Class II KDO8P synthase, a major objective of the present study was to understand the nature of the metal ions at the active site because different metals may have different affinities and coordination geometry toward protein ligands. An ancillary goal was to define the kinetic reaction pathway to serve as a basis for ordination geometry toward protein ligands. An ancillary goal because different metals may have different affinities and co-

KDO8P synthases, a major objective of the present study was to characterize a Class II KDO8P synthase. The role of metal ion in catalysis as well as functional similarities and differences with the metal-independent KDO8P synthases are discussed.
the concentration of A5P was kept constant at a saturation level. To maintain a saturating level of A5P experiments were performed at 300 μM of A5P (Kₘ of A5P ~ 39 μM). The initial rate was calculated from a linear least square fit of the decrease in the absorbance of the curve. As expected, the experimental data under the above conditions were attempted to fit typical Michaelis-Menten kinetics (see “Results” and “Discussion”). This analysis allows determination of apparent Michaelis constant (Kₘ) and maximum velocity (Vₘₐₓ) of the enzyme activity.

Preparation of H. pylori ApoKDO8P Synthase—200 μM of H. pylori KDO8P synthase solution was prepared using 50 mM Tris-HCl, pH 7.5. The enzyme was incubated with freshly prepared EDTA (final concentration of EDTA was kept 10 times higher than the enzyme) and kept in ice for about 45 min. Then the activity assay of the EDTA-treated enzyme was carried out and found that the enzyme has almost 3–4% activity. The solution was then dialyzed against two changes of 4 liters of 50 mM Tris-HCl, pH 7.5, at 4°C.

Preparation of Metal-reconstituted H. pylori KDO8P Synthase—Cd²⁺-reconstituted KDO8P synthase enzyme was prepared by incubating the apoenzyme (200 μM, final concentration) with CdCl₂ (concentration of CdCl₂, was kept 8 times higher than the apoenzyme) in 50 mM Tris-HCl, pH 7.5, and the mixture was kept in ice for about 30 min. A quick activity assay was done with the CdCl₂-treated apoenzyme, and it was found that the enzyme has good activity. The mixture was then dialyzed against two changes of 4 liters of 50 mM Tris-HCl, pH 7.5, at 4°C. The level of Cd²⁺ present in the dialyzed sample was determined by electron beam ionization mass spectroscopy, and the analysis showed that 1 mol of Cd²⁺ was bound with 1 mol of the enzyme (data not shown).

The Cu²⁺, Co²⁺, Mn²⁺, and Ni²⁺ enzymes were prepared in a slightly different manner. In these cases, steady-state assays were performed by incubating the apoenzyme with different metal chlorides, and the initial rates were determined at a fixed concentration of apoenzyme with varying concentrations of metal chlorides. The ratio of metal/apoenzyme corresponded to the maximum initial rate (insets of Fig. 4, A and B, are shown as representative figures) was used for further steady-state and pre-steady-state kinetic experiments.

Rapid Chemical Quench Experiments—Rapid quench experiments were performed with a Kinetik RQ-3 Rapid Chemical Quench (Kinetik Instruments, Austin, TX) as previously described (32). The reaction was initiated by mixing the enzyme solution (15 μl) with the radiolabeled substrate PEP (15 μl). In all cases the concentrations of the enzyme, substrate, and metal cited are those after mixing and during the reaction. The reaction was then quenched with 67 μl of 0.6 N KOH. It is found that the substrate and products are stable under these conditions. The substrates and products were separated and quantified using anion exchange column coupled with simultaneous radioactivity detection.

The HPLC separation was performed on a Mono Q (HR 5/5) anion exchange column with flow rate of 1 ml/min. A gradient separation was employed using solvent A (20 mM triethylammonium bicarbonate, pH 9.0) and solvent B (1 M triethylammonium bicarbonate, pH 9.0) in a linear gradient program of 100–0% A from 0 to 30 min and 0–100% B from 0 to 30 min followed by re-equilibration. The elution times were 15 and 17.8 min for KDO8P and PEP, respectively. During the HPLC analysis a small amount of cold PEP was always injected as an internal standard because it co-elutes with hot PEP and its elution time can be monitored at 292 nm by UV detector. These conditions were used to analyze the samples generated from the rapid chemical quench experiments.

RESULTS

H. pylori KDO8P Synthase Does Not Contain Tightly Bound PEP—Previous studies show that E. coli KDO8P synthase, a Class I KDO8P synthase, was isolated with 1 eq of tightly bound PEP, which contributes stability to the enzyme (33). To determine whether H. pylori KDO8P synthase retained any tightly bound substrates after purification the enzyme was preincubated with either radiolabeled A5P or PEP in the absence of the second substrate. Unlike the Class I KDO8P synthases, no radiolabeled KDO8P peak was found in the HPLC analysis, indicating that this prototypical Class II enzyme does not contain tightly bound PEP substrate. However, reconstitution experiments adding metal to prepare a holoenzyme from the frozen apoenzyme yielded enzyme with a very low specific activity. We were also unable to fully reconstitute the holoenzyme if the apoenzyme was kept for a longer period (2–3 days).

at 4°C. These results indicate that the apoenzyme is less stable upon removal of the metal ions from the native enzyme.

Steady-state Kinetics of the Native and Metal-reconstituted Enzymes—The steady-state kinetic experiments of KDO8P synthase-catalyzed reaction were carried out by monitoring the decrease in the absorbance of PEP at 232 nm. Fig 1A shows the steady-state plot of the native enzyme. The data could be best fitted to a sigmoidal equation yielding Kₘ, kₘₐₓ, and Hill coefficients (n) 2.6 μM, 0.3 s⁻¹, and 2.6, respectively, at pH 7.8. The values of Kₘ, kₘₐₓ were found in good agreement with the reported values (15). The data of Fig. 1A was fitted to a hyperbolic equation as shown in inset of Fig. 1A. Fig. 1, B–D, showed the plots of initial rate versus the concentration of PEP for the Cd²⁺, Cu²⁺, and Co²⁺ enzymes, respectively.Insets of Fig. 1, B–D, show the data were fitted to a hyperbolic equation. However, the same data could be best fitted to a sigmoidal equation yielding n = 4, 2.5, and 2 for Cd²⁺, Cu²⁺, and Co²⁺ enzymes, respectively. The various steady-state kinetic parameters are summarized in Table I.

pH Dependence of the Native and Cd²⁺-reconstituted Enzymes—The pH-dependent experiments of the native and Cd²⁺-reconstituted enzymes were carried out in the pH range 6–9.5. Vₘₐₓ and Kₘ were determined at each pH, and the data were shown in Fig. 2, A and B. For the native enzyme both Vₘₐₓ and Kₘ exhibited bell-shaped pH dependence. Again the plot of the second order rate constant (Vₘₐₓ/Kₘ) also showed similar pH dependence. A fit of the experimental data of Fig. 2A (for Vₘₐₓ/Kₘ) to the equation Vₘₐₓ/Kₘ = (Vₘₐₓ/Kₘ)₀/[1 + (H⁺/[K₉) + (K₉')]) gave K₉ = 9 and K₉' = 5.8, where K₉ and K₉' are the acid equilibrium constants. However, for Cd²⁺ enzymes, K₉ was found to be lower. For Cd²⁺ enzymes, Vₘₐₓ was found to be lower at pH 7 maximum, and Vₘₐₓ showed a single ionization. For Cd²⁺ enzymes the decrease in the Vₘₐₓ/Kₘ with pH (Fig. 2B) could be best fitted to an equation Vₘₐₓ/Kₘ = (Vₘₐₓ/Kₘ)₀/[1 + (H⁺/[K₉) + [K₉']]), yielding K₉' = 7.5.

Pre-steady-state Burst Experiments with Native (Zn²⁺) and Metal-reconstituted Enzymes—Transient-state kinetic methods allow definition of the sequence of reactions occurring at the active site of an enzyme after substrate binding and leading to product release. On the other hand, steady-state kinetic analysis only establishes the order of substrate binding and the order of product release; the steady-state kinetic parameters kₘₐₓ and kₘₐₓ/Kₘ only define the maximum rate of substrate to product conversion and a lower limit for the rate of substrate binding, respectively. Steady-state kinetic analysis cannot address questions regarding the pathway of events occurring at the active site after substrate binding and before product release. To identify the rate-limiting step of the catalysis, we have carried out pre-steady-state burst experiments using radiolabeled [¹⁴C]PEP as a substrate to determine the rate of formation of radiolabeled product [¹⁴C]KDO8P. In this type of experiment the radiolabeled substrate is used in slight excess over enzyme such that the first enzyme turnover as well as multiple turnovers can be examined. A representative burst experiment of the native enzyme showing the time course for biphasic formation of [¹⁴C]KDO8P is shown in Fig. 3A. The rate of the product formation was ~0.8 s⁻¹ for the fast phase and 0.3 s⁻¹ for the slower linear phase. Fig. 3B shows the similar burst experiment for the Cd²⁺ enzyme. The data were fitted to a burst equation yielding rate constants 88 and 1.1 s⁻¹ for the fast and slow phases, respectively. The rate constant of the slow phase for both the native and Cd²⁺ enzymes correspond to the steady-state rate (kₘₐₓ) at 24°C. The amplitude of the burst experiment shown in Fig. 3A provides an estimate of active site concentration, which was determined to be ~20% for the native enzyme. However, it was found to be 55% for the Cd²⁺ enzyme. Fig. 4, A and B, represent the burst experiments of Cu²⁺ and
Co²⁺ enzymes showing a fast burst phase followed by a steady-state phase. The rate for the burst and steady-state phases of Cu²⁺ and Co²⁺ enzymes was found to be 1.7 and 0.3 s⁻¹ and 16.6 and 1.6 s⁻¹, respectively. The active site concentrations of Cu²⁺ and Co²⁺ enzymes were found to be 79 and 29%, respectively. However, no burst of product formation was observed in case of Mn²⁺ and Ni²⁺ enzymes, indicating that the product release is not the rate-limiting step for the catalytic reaction (Fig. 4C).

**DISCUSSION**

Our studies have focused on using *H. pylori* KDO8P synthase as a prototypical metalloenzyme belonging to the Class II KDO8P synthase family, providing a basis for comparison to

**TABLE I**

*Steady-state kinetic parameters of KDO8P synthases*

| Enzyme                        | $K_m$ | $k_{cat}$ | $k_{cat}/K_m$ | Hill coefficient |
|-------------------------------|-------|-----------|---------------|------------------|
| E. coli KDO8P synthase        | 5     | 4.5       | 0.9           | 2.6              |
| *H. pylori* Zn²⁺-KDO8P synthase | 2.6   | 1.0       | 0.19          | 4                |
| *H. pylori* Cd²⁺-KDO8P synthase | 5.8   | 0.1       | 0.017         | 2.5              |
| *H. pylori* Cu²⁺-KDO8P synthase | 7.5   | 0.1       | 0.013         | 2.1              |
the metal-independent Class I KDO8P synthases. The important but unanswered questions were to define the role of the divalent metal ion as well as identify similarities and differences in the kinetic reaction pathway between the two classes of KDO8P synthases.

The metal ion may play a structural and/or catalytic role by modulating protein stability and/or catalysis. An initial suggestion was that the metal ion in the Class II enzymes might play a role as a Lewis acid by coordinating the aldehyde group of A5P, thereby polarizing the carbonyl carbon of aldehyde. Accordingly, this would facilitate the nucleophilic attack by C3 of PEP to the aldehyde of A5P. Structural studies, however, do not support this suggestion. The crystal structure of *A. aeolicus* Cd\(2^+\)/H11001-KDO8P synthase, a Class II thermophilic enzyme, has been solved in several binary complexes with either PEP or A5P substrates (22) as well as a low temperature catalytically inactive ternary complex containing metal and both substrates. In each case it was observed that the 2-OH group of A5P was coordinated with the Cd\(2^+\) ion. In these complexes, the protein ligands, His-185, Asp-233, Glu-222, Cys-11, and a water molecule have been found to coordinate the Cd\(2^+\) ion, giving rise to a distorted octahedral coordination state. In the enzyme-PEP binary complex, two water molecules are located in van der Waals contact with the PEP. Taken together these structural studies suggest that the metal ions may play a direct role in catalysis by activating the water molecule for the nucleophilic attack to the C2 carbon of PEP or may indirectly modulate catalysis by orienting substrates in the optimal geometry within the active site to allow the chemical reaction to occur. This might in turn stabilize a putative hemiketal phosphate intermediate, although it remains to be established whether the metallo-KDO8P synthases use a similar mechanism as established for the *E. coli* KDO8P synthase (see Scheme 1).

Earlier studies have shown that the Class I *E. coli* KDO8P synthase was isolated with 1 eq of bound PEP. This enzyme was less stable upon removal of the bound PEP (33), suggesting that the PEP maintains a critical role in stabilizing the enzyme. More recent studies on the *E. coli* KDO8P synthase from our laboratory suggest this may be mediated by conformational changes in which the removal of bound PEP changes the original conformation of the enzyme at the active site to produce an enzyme that is less active. The native *H. pylori* KDO8P synthase does not have any bound PEP; however, the metal ion may play a similar role since it was observed that the enzyme was much less stable in the absence of metal ion. This may be due to a protective effect of the metal ion that prevents oxidation of the cysteine ligand in the coordination state. Thus, it is likely that the metal ions not only activate the water molecule for the nucleophilic attack but also maintain the stability/original conformation of the enzyme.

As illustrated in Table I, there are distinct differences between Class I and Class II, *E. coli* and *H. pylori*, enzymes, respectively, in terms of their catalytic efficiencies (\(k_{\text{cat}}/K_m\)). Accordingly, the native *H. pylori* enzyme is almost 8-fold less efficient than the *E. coli* enzyme. This is primarily due to variations in the overall rate of catalysis, \(k_{\text{cat}}\), whereas there was little change in \(K_m\) values for Class I and Class II enzymes. The \(k_{\text{cat}}\) of *E. coli* enzyme was determined to be 15-fold higher than the *H. pylori* enzyme. It is interesting to note that the rate of \(k_{\text{cat}}\) for the *H. pylori* Cd\(2^+\) enzyme was found to be 3-fold faster than that of the native enzyme (Zn\(2^+\) enzyme), and

\[^{2}\]Z. Li, A. K. Sau, and K. S. Anderson, submitted for publication.
with the residues involved in catalysis, the effect of pH was examined. The pH dependence of the native and Cd\(^{2+}\) enzymes showed interesting behavior. The pH dependence of \(V_{\max}\) and \(K_m\) for the native enzyme showed a bell-shaped trend. Although an absolute fit to the data in both cases was difficult, apparent inspection gave the approximate \(pK_a\) values of 7.7, 8.5 and 7.8, 8.4 for \(V_{\max}\) and \(K_m\), respectively. Typically, the pH dependence of \(V_{\max}/K_m\) represents the ionization of the free enzyme and free substrate. For the native enzyme containing Zn\(^{2+}\) as the metal ion, the pH dependence of \(V_{\max}/K_m\) gave a good fit (Fig. 2A) with two \(pK_a\) values of 5.7 and 9. The difference in \(pK_a\) values obtained from \(V_{\max}/K_m\) and \(V_{\max}/K_m\) could be due to the difference in the ionization of the same residue in the enzyme-substrate complex and free enzyme. It is unlikely that the substrates (PEP and A5P) have any ionization in this pH range. Hence, these must be attributed to the enzyme. The former \(pK_a\) may correspond to a histidine residue and latter to a cysteine or lysine residue. The \(pK_a\) of the free histidine residue in water is 6.3. Although the observed \(pK_a\) for histidine in this case is slightly lower than the free acid, it is not surprising based upon studies with other enzyme systems. For instance, the \(pK_a\) of histidine in papain and cytotoxic ribonuclease \(\alpha\)-sarcin were reported to be 4.3 and 5.8, respectively (35, 36). In contrast, studies with the Cd\(^{2+}\) enzyme, in which pH is dependent on \(V_{\max}/K_m\), showed only one \(pK_a\) (=7.5). This may correspond to a cysteine residue. Unlike the results obtained for the native enzyme, these data suggest that in Cd\(^{2+}\) enzyme histidine residue may be far away from the active site and, hence, not directly involved in the catalysis.

A transient kinetic approach was used to further understand the kinetic reaction pathway of the Class II \(H.\) pylori enzyme and provide clues as to how catalysis may be occurring at the active site. This involved examining the pre-steady-state burst kinetics using rapid chemical quench methodology. A pre-steady-state burst of formation of KDO\(^8\)P was observed followed by a steady-state phase at a rate close to \(k_{\text{cat}}\). This behavior is indicative of a mechanism in which the chemical catalysis does not limit the overall reaction, but rather, the release of product KDO\(^8\)P is rate-limiting.

In studies with the native enzyme as shown in Fig. 3A, it is clear that the plot has a very shallow burst and a definite positive intercept. Hence, the data were fitted to a burst equation, giving low burst rate (0.8 s\(^{-1}\)). In addition, the steady-state rate (0.3 s\(^{-1}\)) obtained from the burst experiment is in excellent agreement with the \(k_{\text{cat}}\) (0.3 s\(^{-1}\)) determined from steady-state experiments. The amplitude of the burst gives an estimate for the active site concentration of the enzyme and, in the case of the native \(H.\) pylori metallo-KDO\(^8\)P synthase enzyme, this value was determined to be ~20%. This suggests that the enzyme has a low concentration of active sites. However, for the Cd\(^{2+}\) enzyme the burst and steady-state rates were determined to be 88 and 11.1 s\(^{-1}\). This clearly showed that the burst of Cd\(^{2+}\) enzyme is much higher than that of native enzyme. Because there is a faster step that governs catalysis, any enzyme reaction intermediate transiently formed may accumulate during a single enzyme turnover, thus enhancing the opportunity for detection in Cd\(^{2+}\) enzyme. The active site concentration of Cd\(^{2+}\) enzyme was determined to be 55%, more than 2-fold higher as compared with the native enzyme. The steady-state rate (1.1 s\(^{-1}\)) obtained from the burst experiment was also in good agreement with the \(k_{\text{cat}}\) (1.0 s\(^{-1}\)) determined by steady-state experiments.

It is interesting to note that earlier studies with the \(E.\) coli KDO\(^8\)P synthase also revealed a burst of KDO\(^8\)P formation followed by a steady-state was observed (34). The burst rate was found to be 91 s\(^{-1}\). Thus the pre-steady-state kinetics of \(E.\) coli KDO\(^8\)P synthase fits a burst equation with two different rate constants, one for the burst and another for the steady-state phase. This suggests that there is a faster step that governs catalysis, any enzyme reaction intermediate transiently formed may accumulate during a single enzyme turnover, thus enhancing the opportunity for detection in Cd\(^{2+}\) enzyme. The active site concentration of Cd\(^{2+}\) enzyme was determined to be 55%, more than 2-fold higher as compared with the native enzyme. The steady-state rate (1.1 s\(^{-1}\)) obtained from the burst experiment was also in good agreement with the \(k_{\text{cat}}\) (1.0 s\(^{-1}\)) determined by steady-state experiments.
coli KDO8P synthase and H. pylori Cd$^{2+}$-reconstituted KDO8P synthase behave similarly, although these two enzymes evolved to use different strategies for their catalytic reactions. In case of the Cu$^{2+}$ enzyme, a shallow burst was observed with a burst rate 1.7 s$^{-1}$, which is slightly higher than that of the native enzyme. But a higher burst rate (16.6 s$^{-1}$) was observed in case of the Co$^{2+}$ enzyme compared with its native enzyme. The active site concentrations were found to be 79 and 29% for the Cu$^{2+}$ and Co$^{2+}$ enzymes, respectively. Although the active site concentration of the Cu$^{2+}$ enzyme is higher than that of the Co$^{2+}$ enzyme, the burst rate is lower for the Cu$^{2+}$ enzyme than that of Co$^{2+}$ enzyme. Interestingly, no burst of KDO8P formation was observed in case of the Mn$^{2+}$ and Ni$^{2+}$-enzymes, establishing that the conversion of PEP by the Mn$^{2+}$ and Ni$^{2+}$ enzymes has a different rate-limiting step. The size of the metal ions and the nature of the ligands could play an important role as a result of different coordination geometry of the metal ions in the protein environment. For example, Zn$^{2+}$ (ionic radius, 0.74 Å) prefers distorted tetrahedral/tetrahedral/trigonal bipyramidal structure, whereas Cd$^{2+}$ (0.92 Å) and Co$^{2+}$ (0.72 Å) prefer distorted octahedral/octahedral structure. On the other hand, Cu$^{2+}$ (0.71 Å), Mn$^{2+}$ (0.8 Å), and Ni$^{2+}$ (0.69 Å) prefer square planer/trigonal bipyramidal/square pyramidal geometry. The different coordination geometry and/or available ligands of the metal ions may be the reason for the change in active site and rate of chemical catalysis in this enzyme.

In summary, the first detailed kinetic characterization of a metal-dependent Class II KDO8P synthase from H. pylori is presented and serves as a basis of comparison to the metal-independent Class I KDO8P synthases. Important findings regarding the role of metal ions in catalysis in Class II KDO8P synthases include (i) a marked difference in catalytic efficiency that is dependent upon the metal ion with Cd$^{2+}$, showing the highest activity and specificity compared with the other metals, ii) cooperative behavior for the binding of PEP in the native as well as the other metal-reconstituted enzymes, with the highest degree of cooperativity in the case of Cd$^{2+}$ (n = 4), (iii) versatility of different metal ions, illustrated as the rate-limiting step changes, dependent upon the particular divalent metal ion bound (product release is the rate-limiting step for the native, metal-reconstituted enzymes, but for the Mn$^{2+}$ and Ni$^{2+}$-enzymes, chemistry is rate-limiting), and iv) a rate of chemical catalysis that is higher for Cd$^{2+}$ than for the other metal ions.

A comparison of the kinetic reaction pathway for Class I and Class II KDO8P synthases reveals that for each class the rate-limiting step, release of the KDO8P product, is in general the same (noting that the Mn$^{2+}$ and Ni$^{2+}$ metalloenzymes are exceptions). Also the presence of either a divalent metal in the Class II KDO8P synthases or tightly bound PEP substrate in the Class I KDO8P synthases promotes stability. A marked difference in the two classes is the observation of cooperative behavior for the Class II KDO8P synthases. The present study illustrates how two different classes, one metal dependent and
one metal-independent, have evolved to carry out the same catalytic reaction using similar active site structural architecture. The divalent metal ions in the Class II KDO8P synthases play dual roles by modulating catalysis and affecting protein quaternary structure.

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