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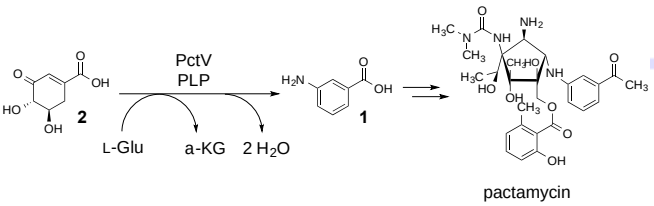
Mechanism-based Trapping of the Quinonoid Intermediate Using the K276R Mutant of PLP-Dependent 3-Aminobenzoate Synthase PctV in the Biosynthesis of Pactamycin

Akane Hirayama,[a] Akimasa Miyanaga,[b] Fumitaka Kudo*[b] and Tadashi Eguchi*[a]

Abstract: Mutational analysis of the pyridoxal 5′-phosphate (PLP)-dependent enzyme PctV has been carried out to elucidate the multi-step reaction mechanism for the formation of 3-aminobenzoate (3-ABA) from 3-dehydroshikimate (3-DSA). The results revealed that the introduction of a K276R mutation led to the accumulation of a quinonoid intermediate with an absorption maximum of 580 nm following the reaction of pyridoxamine 5′-phosphate (PMP) with 3-DSA. Furthermore, the chemical structure of this intermediate was supported by X-ray crystallographic analysis of the complex formed between the K276R mutant and the quinonoid intermediate. These results clearly show that a quinonoid intermediate is involved in the formation of 3-ABA, and also indicate that the Lys276 residue in the active site of the PctV enzyme plays multiple roles including acid/base catalysis during the dehydration reaction of the quinonoid intermediate, as well as assisting in the PMP formation and aminotransfer reaction.

Introduction

PctV is a pyridoxal 5′-phosphate (PLP)-dependent enzyme, which catalyzes the formation of 3-aminobenzoic acid (**1**, 3-ABA) from 3-dehydroshikimic acid (**2**, 3-DSA) during the biosynthesis of antitumor antibiotic pactamycin (Scheme 1).[1] Given that isotope-labeled 3-ABA has been shown to be efficiently incorporated into pactamycin in feeding experiments, it seems plausible that the PctV reaction initiates this particular secondary metabolic pathway.[1a,2] PctV shows a moderate level of similarity to glutamate-1-semialdehyde-2,1-aminomutase (GSAM, Figure S1), which is involved in the formation of 5-aminolevulinic acid from glutamate-1-semialdehyde during heme biosynthesis.[3] Thus, PctV belongs to the aminotransferase family of enzymes, which possess a critical lysine residue in their active site that forms an internal aldimine complex with PLP (Scheme S1). The enzyme-PLP aldimine complex formed in the active site of PctV can react with a free amino acid to form an external aldimine followed by a ketimine intermediate, which can be hydrolyzed to give pyridoxamine 5′-phosphate (PMP) and α-ketoacid. PctV uses L-glutamate to allow for the formation of PMP.[1a] The amino group of the PMP generated in this way can then behave as a nucleophile and attack the carbonyl carbon atom of 3-DSA to form a ketimine intermediate. In a typical aminotransfer reaction, this ketimine intermediate is converted to an aldimine intermediate, which undergoes nucleophilic attack by the active site lysine residue to give the initial enzyme-PLP aldimine. The aminated product is then released from the enzyme. In the case of the PctV reaction, additional dual dehydration steps after the aminotransfer reaction would be required prior to the release of the 3-ABA product (Scheme 1). Thus, specific catalytic reaction mechanisms appear to be required for the dehydration stages of the PctV-catalyzed reaction. Given that a better understanding of the underlying mechanism of this dehydration process could be used to expand the repertoire of PLP-dependent enzymatic chemistry,[4] we recently became interested in the details of the PctV reaction mechanism.



Scheme 1. Formation of 3-aminobenzoate from 3-dehydroshikimate by PctV during the biosynthesis of pactamycin.

Results

Model Structure and Mutational Analysis of PctV. A Swiss model[5] structure of PctV based on GSAM (36% identity to PctV) with the gabaculine-PLP complex[6] clearly showed that Lys276 is the key lysine residue in the active site (Figure S2). Several other residues, including Tyr64, Lys159, Asn220, and Glu416 were located in close proximity to the gabaculine-PLP complex in the original structure, which indicated that some of these residues could be involved in the recognition of 3-DSA, as well as assisting in the dehydration reactions. However, kinetic analysis of the Y64F, K159Q, N220A, and E416A mutants revealed that their k_{cat} values (0.18–0.28 min^{-1}) were similar to that of the wild type enzyme (0.39 min^{-1}) (Table 1). This result therefore indicated that these amino acid residues are not involved in the catalytic reactions, although Lys159 and Asn220 appear to be involved in the recognition of the substrate because the K_M values for the K159Q and N220A mutants were

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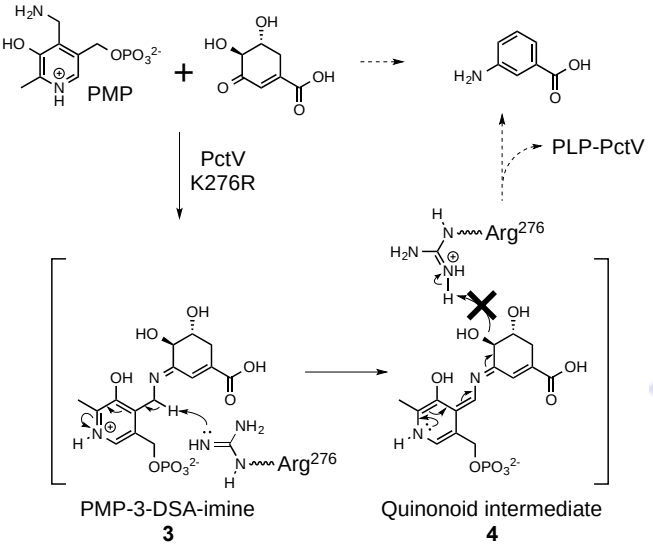
7- and 62-fold higher than that of the wild type (2.1 μM), respectively.

Table 1. Kinetic values of PctV and its mutants.

	Wild type	Y64F	K159Q	N220A	K276Q	E416A
K_M [μM]	2.1 ± 0.4	1.5 ± 0.1	15 ± 2	130 ± 30	N. D. [a]	1.6 ± 0.4
k_{cat} [min^{-1}]	0.39 ± 0.04	0.28 ± 0.02	0.18 ± 0.02	0.28 ± 0.06	N. D. [a]	0.20 ± 0.01

[a] N. D. means not detected. PctV (0.1 μM) was reacted with L-glutamate (200 mM), PLP (100 μM), and 3-DSA at 28 °C. Kinetic values were estimated by Michaelis-Menten plot.

As expected, the K276Q and K276R mutants did not afford detectable amounts of 3-ABA from 3-DSA (data not shown), which provided further confirmation that Lys276 is critically important to the formation of a PLP-lysine aldimine complex and the subsequent generation of PMP with L-glutamate. During the aminotransfer reaction between PMP and an amino acceptor, the lysine residue would act as a base to abstract a proton from the ketimine intermediate of the PMP-amino acceptor complex to give a quinonoid intermediate (Scheme S1). The resulting ε-ammonium moiety of the lysine residue would then act as an acid to supply a proton for the formation of an aldimine intermediate, which would undergo nucleophilic attack by the amino group of the lysine residue to give the initial internal PLP-aldimine complex. Notably, the reaction of the K276Q mutant with PMP and 3-DSA only resulted in the formation of PMP-3-DSA **3**, which was formed in the absence of enzyme (Figure S3). Therefore, this result indicated that the glutamine residue in the K276Q mutant does not act as base and thus the deprotonation of **3** did not occur. In contrast, the reaction of the K276R mutant with PMP and 3-DSA led to the accumulation of a reddish-purple reaction intermediate, which gave a strong ultraviolet/visible (UV/Vis) absorption at 580 nm (Figure 1). This result indicated that the arginine residue in the K276R mutant was acting as a base like as Lys276 in the wild type to abstract a proton from the PMP-3-DSA ketimine complex **3** to give a quinonoid intermediate **4**, as evidenced by the long wavelength of the absorption (Scheme 2). The results of an *ab initio* calculation using the Gaussian 09 program to determine the structure of the reaction intermediate based on its UV/Vis absorption spectrum supported the idea that a quinonoid intermediate was formed prior to the dehydration step (Figure S4). Given that the acidity of the guanidinium ion resulting from the protonation of the arginine residue would be weaker than that of the lysine ammonium ion, it is unlikely that the arginine residue would behave as an acid catalyst and provide a proton for the subsequent dehydration reaction. This would therefore lead to the observed accumulation of the quinonoid intermediate **4** in the active site (Scheme 2).



Scheme 2. Proposed mechanism for the reaction of PMP with 3-DSA in the presence of the K276R mutant

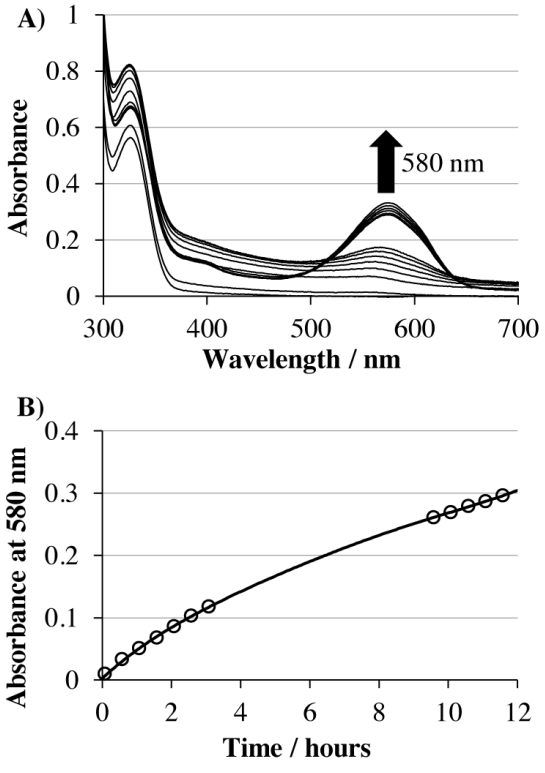


Figure 1. K276R (40 μM) reaction with PMP (40 μM) and 3-DSA (400 μM) at room temperature. A) The UV/Vis spectra were measured at 30-min intervals (0–3 hr and 9.5–12 hr). B) Time course for the absorbance at 580 nm.

Crystal Structure Analysis. To determine the structure of the intermediate accumulated during the reaction of PMP with 3-DSA in the presence of the K276R mutant, we solved the X-ray crystal structure of the complex bound to the K276R mutant (2.4 Å resolution), as well as the wild type enzyme with PLP (Figure 2, Figure S5, Table S1). The overall structure of PctV is similar to that of GSAM, as expected from the model structure (Figure S5). The X-ray crystal structure of the wild type revealed that PctV exists as a homodimer in its crystal form as well as in solution (data not shown), and that the active site Lys276 residue is positioned at the dimer interface. The crystal structure of the K276R mutant showed a significant electron density in the active site corresponding to the structure of the PMP-3-DSA complex (Figure 2B). The Asn220 residue was found to be located in close proximity to the C-4 hydroxy group of 3-DSA (2.6 Å) and the hydroxy group of PLP (2.8 Å), which indicated that this residue is involved in the recognition of these two substrates. The carboxylate moiety of the 3-DSA substrate forms interactions with the Ser28 and Arg31 residues of the enzyme. Furthermore, the six-membered ring structure derived from 3-DSA contained two hydroxy groups, which indicated that the accumulated intermediate was a ketimine or quinonoid intermediate of PMP-3-DSA. However, the electron density corresponding to the hydroxy groups was relatively weak, most likely because of the partial dehydration of the intermediate during X-ray irradiation. Taken together, these results suggested that the K276R mutant trapped the quinonoid intermediate **4** because of the low acidity (*pK*a = 12.5 in aqueous solution) of the resulting guanidinium ion, which would prevent the occurrence of the subsequent dehydration reaction (Scheme 2). Thus, the Lys276 residue in the wild type enzyme would act as a base to allow for the formation of the quinonoid intermediate **4** with the concomitant formation of the ε-ammonium ion of the lysine residue (*pK*a = 10.7 in aqueous solution), which would act as an acid catalyst to facilitate the dehydration of the intermediate to give the corresponding aldimine intermediate **6** (Scheme 3).

Additional Mutational Analysis. The structure of the PMP-3-DSA-K276R complex revealed that the Asn220, His411, and Glu416 residues could interact with the C-5 hydroxy group of the reaction intermediate through two water molecules and therefore seemed to be involved in the second dehydration step (Figure 2). However, the H411A, H411A/E416A, and N220A/H411A/E416A mutants allowed for the efficient production of 3-ABA from 3-DSA with PLP and L-glutamate (Figure S6), which indicated that these residues are not important to the dehydration reaction. Thus, it is still unclear whether the second dehydration occurs enzymatically or non-enzymatically. The ε-amino group of the Lys276 residue might attack the aldimine intermediate **6** to promote the second dehydration reaction and the formation of the ketimine intermediate **7** (Scheme 3). Last, deprotonation of the C-6 position and aromatization, followed by the formation of an internal PLP-lysine aldimine would give 3-ABA **1** as the final product.

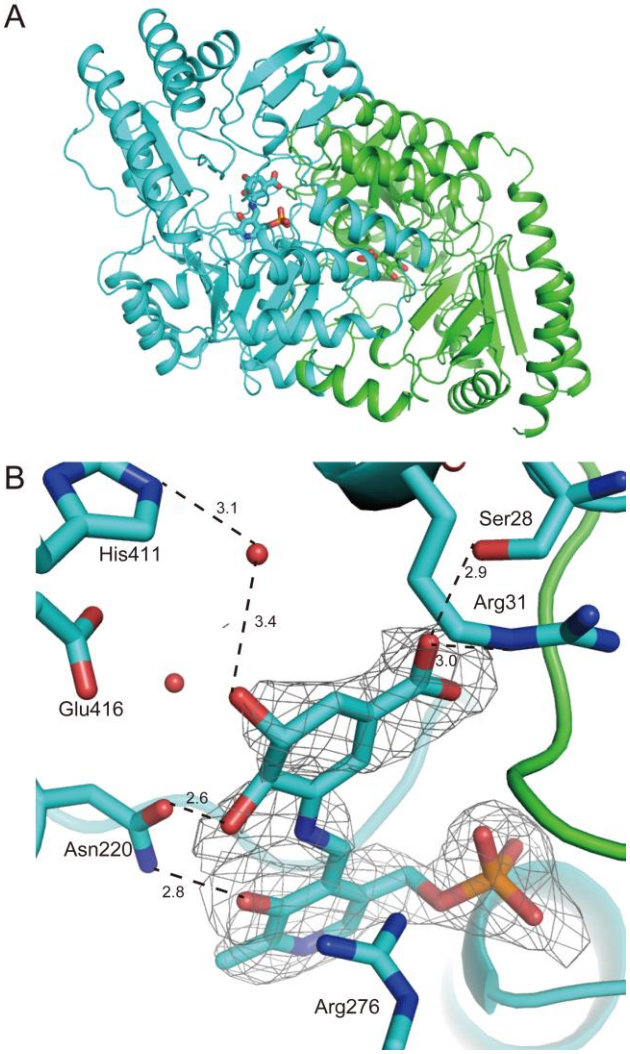
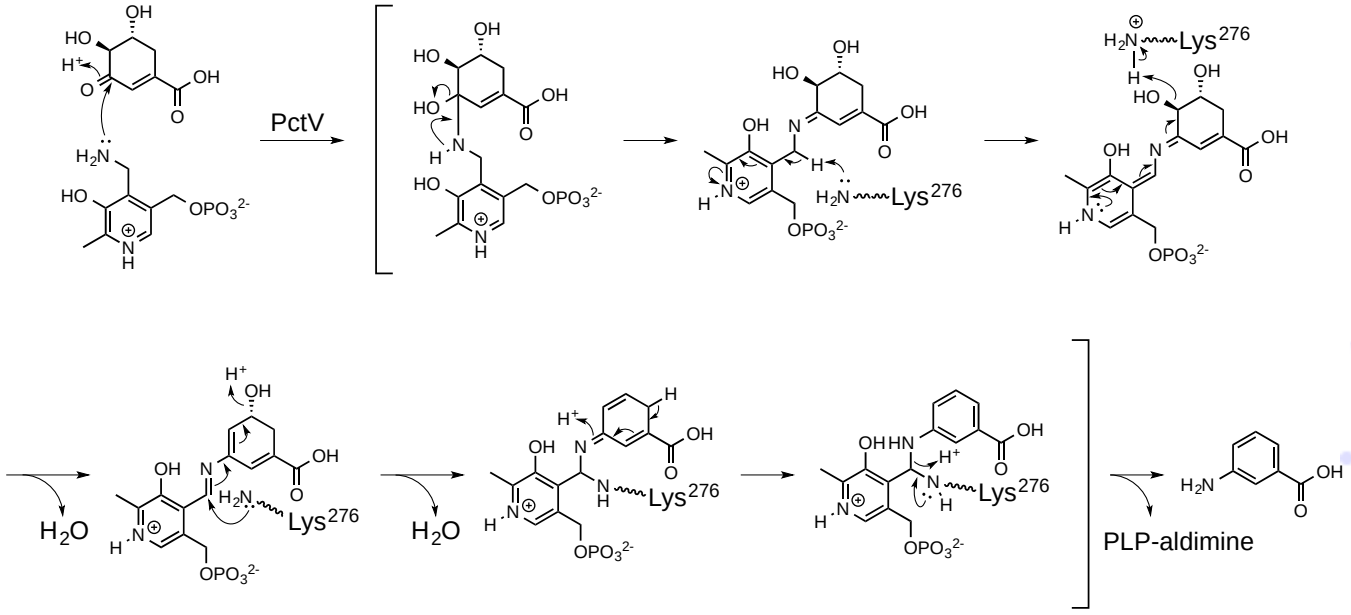


Figure 2. Crystal structure of K276R with PMP-3-DSA. (A) Overall structure, (B) active site with the PMP-3-DSA. Monomeric subunits are colored in green and cyan. An F_o-F_c electron density map contoured at 2.5 σ was constructed prior to the incorporation of the PMP-3-DSA molecule. Interactions with PMP-3-DSA are shown as broken lines.

The reaction of the R31A mutant with L-glutamate, PLP and 3-DSA did not afford 3-ABA, whilst the same reaction with the S28A mutant produced 3-ABA, albeit at much slower rate than the wild type enzyme (data not shown). It was difficult to perform kinetic analysis experiments using the S28A mutant, because the K_M value for 3-DSA seems to be too high. In contrast, both the S28A and R31A mutants reacted with PMP and 3-DSA to produce 3-ABA with similar efficiency to the wild type enzyme (Figure S7). However, the production rates of PMP from PLP and L-glutamate were very slow for the S28A and R31A mutants (Figure S8). These results therefore suggested that the Ser28 and Arg31 residues are involved in the recognition of the substrates, presumably via the carboxylate moieties of L-glutamate and 3-DSA, rather than the dehydration steps.



Scheme 3. Proposed reaction mechanism of 3-ABA formation by PctV.

Stopped-flow Analysis with Wild Type of PctV. Notably, stopped-flow analysis in the reaction of the wild type enzyme with PMP and 3-DSA revealed that the same quinonoid intermediate with an absorption band at 580 nm appeared to be transiently generated (Figure 3). Therefore, it was concluded that this quinonoid intermediate **4** is a genuine intermediate in the PctV reaction (Scheme 3). The formation rates of the quinonoid intermediate **4** (580 nm) by wild type of PctV and K276R mutant were estimated to be 24 AU/min and 6.7 x 10^{-4} AU/min, respectively (Figure 1 and 3). This result indicates that the K276R mutation significantly reduces the reaction rates before the quinonoid formation, whilst the Arg residue of the mutant could work as base like as Lys276 in the wild type to generate the quinonoid intermediate **4**. Since probable PMP-3-DSA-imine intermediate **3** was not observed in the K276R reaction with PMP and 3-DSA, the initial nucleophilic attack of the amino group of PMP to the ketone of 3-DSA would be affected by this mutation. Thus, ε-ammonium of the Lys residue in the wild type might work as an acid catalyst to accelerate the initial nucleophilic addition during the formation of the ketimine intermediate **3**. It is revealed that the ketimine intermediate **3** is slowly generated in the K276Q reaction with PMP and 3-DSA (Figure S3). Thus, in the K276R reaction with PMP and 3-DSA, the ketimine intermediate **3** might be non-catalytically generated and the Arg residue might work just as a base catalyst to form the quinonoid intermediate **4**.

The disappearance rate of the quinonoid intermediate **4** (580 nm) in the wild type was estimated to be ca. 18 min^{-1} by assuming that the dehydration of the quinonoid intermediate is a unimolecular reaction (Figure S9). This rate does not account for the k_{cat} value 0.39 min^{-1} in the steady state kinetics. Therefore,

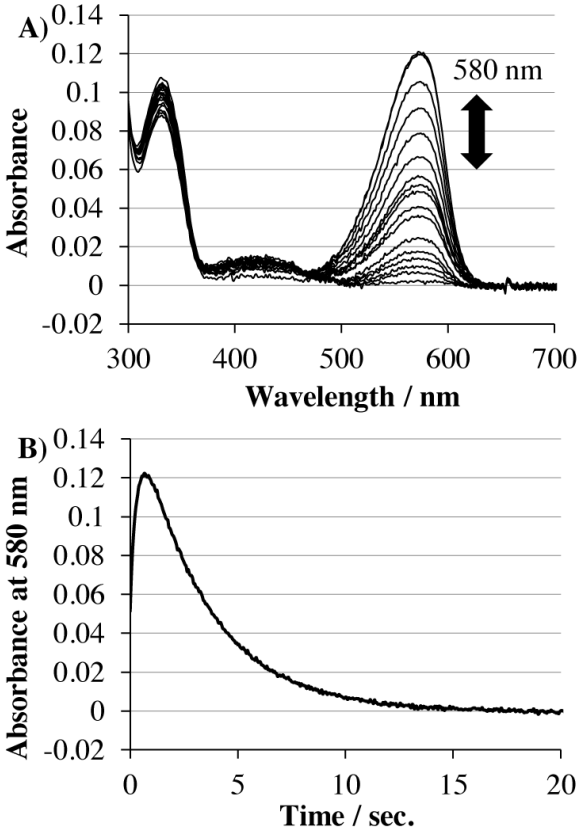


Figure 3. Stopped-flow analysis of the wild type PctV (50 μM) reaction with PMP (50 μM) and 3-DSA (200 μM). A) The UV/Vis spectra were measured at 0.5-sec intervals (0-5 sec) and 1-sec intervals (5-10 sec). B) Time course for the absorbance at 580 nm.

the rate-limiting step seems to exist after the consumption of the quinonoid intermediate. In parallel with the disappearance of the absorption at 580 nm, the absorption at 330 nm and 415 nm appeared to be increased to indicate that subsequent reaction intermediates and/or products were formed before the release of 3-ABA (data not shown). However, it is hard to distinguish several potential intermediates only from the obtained data, because the *ab initio* calculation indicates that those absorptions are overlapped (Figure S4). The aromatization reaction of PLP-dihydro-ABA-aminal intermediate **7** that would show absorption at 300 nm and 400 nm could proceed spontaneously, because any amino acid residue does not interact with this region to abstract a proton judging from the crystal structure of K276R-quinonoid complex (Figure 2B). Thus, because only obscure spectral change to indicate accumulation of reaction intermediates was observed after the disappearance of the quinonoid intermediate, we think that the product release would be very slow and is the rate-limiting step in this reaction.

Discussion

Mechanistic Comparison with ColD. ColD has been reported to catalyze the mechanistically related PLP-dependent dehydration reaction of GDP-4-keto-6-deoxymannnose to give GDP-colitose in the biosynthesis of the *O*-antigen lipopolysaccharide of a Gram-negative bacteria (Scheme S2).[7] ColD has a histidine residue in its active site instead of lysine, therefore the PMP cofactor is an important requirement for this reaction.[8] In the ColD reaction, the histidine residue in the active site abstracts a proton from the PMP-substrate ketimine intermediate to assist in the dehydration step, with the abstracted proton going on to act as an acid catalyst. The switching of the active site histidine residue in ColD to lysine (i.e., H188K) in addition to the introduction of a S187N mutation resulted in a change in the enzymatic function of the enzyme from a dehydratase to an aminotransferase.[9] This change in the function of the mutant ColD enzyme suggested that the lysine in the active site was acting as a base, and that the resulting protonated lysine was so far away from the dehydration site that the enzyme could only facilitate the aminotransfer reaction. These results therefore demonstrate that the positioning of the active site amino acid residue (i.e., histidine or lysine) in the ColD enzyme dictates the enzymatic reaction pathway. This mechanism could also apply to the PctV-catalyzed reaction, where the active site Lys276 residue adjacent to the C-4 hydroxy group of the 3-DSA substrate acts as an acid/base catalyst to catalyze the dehydration of the PMP-3-DSA intermediate. Thus, the location of the lysine residue is critical to determining the reaction path. The K276H mutant of PctV behaved as the K276Q mutant when PMP and 3-DSA were reacted (data not shown). Thus, the His residue of the K276H mutant does not work as a base catalyst to abstract a proton of PMP-3-DSA-imine **3** to give quinonoid **4**, presumably due to the shorter length of the side chain than those of Arg and Lys.

Crucial Role of Catalytic Lysine Residue in PLP-dependent Enzyme. The catalytic importance of the active site lysine residue has been highlighted in several other studies concerning the mutational analysis of several other PLP-dependent enzymes.[10] Once the lysine residue is mutated to another amino acid in these enzymes, PMP is required to allow for the analysis of their enzymatic reactions. Mutant enzymes containing an arginine residue instead of a lysine usually showed much lower levels of activity than the corresponding wild type enzymes.[11] The results of these studies have therefore allowed for the multiple functions of active site lysine residues to be fully characterized.[12] However, there have been no report providing clear evidence to show that the ε-amino group of the lysine residue is critical to the formation of the quinonoid intermediate or that the resulting ε-ammonium ion acts as an acid catalyst to provide a proton for the conversion of the quinonoid intermediate to an aldimine intermediate. The arginine residue in the K276R complex with PMP-3-DSA was found to be located in close proximity to the C-4 hydroxy group of the 3-DSA substrate (3.7 Å, Figure 2B). Thus, in the reaction of the wild type PctV enzyme, the protonation of the C-4 hydroxy group of the quinonoid intermediate **4** appears to occur at the active site lysine residue, followed by the dehydration (Scheme 3). Further, it is suggested that the catalytic lysine would be involved in the PMP-3-DSA-imine **3** formation as an acid catalyst, because the formation rate of the quinonoid intermediate **4** between the wild type and K276R was significantly different.

The formation of the quinonoid intermediate in the PLP-dependent enzymatic reaction has been proposed in several other studies.[13] From the structure of the PctV K276R mutant-PMP-3-DSA complex, we could clearly observe the formation of the native quinonoid intermediate structure during the formation of 3-ABA. This result therefore confirmed that the pyridine ring of PLP/PMP acts as a transient electron sink. The subsequent acid-catalyzed flow of electrons from the electron-rich pyridine ring is therefore the most likely mechanism for the dehydration reaction (Scheme 3). In this sense, the acid-base properties of lysine would be critical to controlling the formation of the quinonoid intermediate and the subsequent protonation to assist the dehydration. In the reaction of the K276R mutant with PMP and 3-DSA, the quinonoid intermediate was accumulated as the only product and any other reaction intermediate was not detected. This result therefore demonstrated that the lysine residue of the PctV enzyme is critical to the dehydration reaction and the formation of 3-ABA.

Conclusions

We have successfully trapped a native form of the quinonoid reaction intermediate formed during the PctV-catalyzed conversion of 3-DSA to 3-ABA by introducing a K276R mutation in the active site of the enzyme. Furthermore, we have provided clear evidence that the Lys276 residue in the active site is critical to the dehydration of the quinonoid intermediate, as well as the formation of the initial PLP-lysine aldimine and PMP-3-DSA- imine intermediates during the PctV-catalyzed formation of

3-ABA. It is envisaged that these results will be used to expand the scope of PLP-dependent enzymatic chemistry.

Experimental Section

Materials and Instruments. All of the biological experiments including DNA manipulation and enzyme treatments were performed according to the standard protocols.[14] The restriction enzymes and ligase were purchased from Takara (Shiga, Japan). DNA oligonucleotide primers were synthesized by Fasmac (Kanagawa, Japan). KUBOTA 3700 (Kubota, Tokyo, Japan) and HIMAC CENTRIFUGE SCR20B (Hitachi, Tokyo, Japan) centrifuges were used for centrifugation. UV/Vis spectral analyses and absorbance measurements were recorded on a Shimadzu UV-2450 spectrophotometer (Kyoto, Japan). Stopped flow analysis were performed using FS-100 high-speed scan spectrophotometer (JASCO, Tokyo, Japan), SFS-472 stopped flow system (JASCO, Tokyo, Japan), and ED Heating Immersion Circulator (JULABO, Seelbach, Germany).

Mutational Analysis of PctV. Mutagenesis of *pctV* was performed by QuikChange® method. The plasmid *pctV*/pET28, which was obtained in previous report[1a], was used as template for PCR. Sequences of used primers were listed in Table S2. PCR conditions: 94 °C for 2 min 12 or 16 cycles of 98 °C for 10 s, 60 °C for 30 s, and 68 °C for 3.5 min; 0.2 μL of KOD -plus- Neo polymerase (TOYOBO, Osaka, Japan), 1.0 μL of 10× PCR Buffer for KOD -plus- Neo, 1.0 μL of dNTPs (2 mM each), 0.6 μL of $MgSO_4$ (25 mM), 0.3 μL of primer-F (10 μM), 0.3 μL of primer-R (10 μM), 0.5 μL of DMSO, 10 ng of template, adjusted to 10 μL with water. 5 μL of PCR reaction mixture was used for checking amplification and another 5 μL was added 0.1 μL of *Dpn*I for digestion of template DNA. After incubation at 37 °C, 2 μL of reaction mixture was used for transformation of *E. coli* DH5. Transformant was cultured on lysogeny broth (LB) containing kanamycin (30 μg/mL). Several colonies were cultured and plasmid DNA was extracted. After confirming its sequence, *E. coli* BL21(DE3) was transformed by the plasmid and cultured on LB containing kanamycin (30 μg/mL). H411A/E416A variant and N220A/H411A/E416A variant were made using almost the same method with *pctV*-H411A/pET28 and *pctV*-H411A-E416A/pET28 as template, respectively. All recombinant proteins were expressed and purified according to the previously described method.[1a]

To test enzymatic activity, PctV mutants were reacted with L-glutamate, PLP, and 3-DSA at 28 °C. Reaction mixtures contained 1 μM of PctV mutant, 100 μM of PLP, 200 mM of L-glutamate, and 200 μM of 3-DSA (total 50 μL). Enzymatic reactions were quenched by adding 50 μL methanol. After centrifugation (15,000 rpm, 30 min), supernatants of these samples were filtered through Millex LG filters (Millipore, Darmstadt, Germany). The obtained solutions were then injected into an HPLC instrument (Elite LaChrom L-2455 DAD Detector and L-2130 Pump, Hitachi, Tokyo, Japan) equipped with a Phenomenex® Luna 3 μ PFP(2) 100 Å 150 × 4.6 mm column (Phenomenex, California, USA). The elution conditions were 100 mM NaH_2PO_4-5% CH_3CN-H_2O, at a flow rate 0.5 mL/min at room temperature. Some PctV variants were also reacted with PMP and 3-DSA at 28 °C. Reaction mixtures contained 10 μM of PctV variant, 100 μM of PMP, and 200 μM of 3-DSA (total 50 μL). HPLC analyses were performed as described above.

Kinetic Analyses of PctV Mutants. Tris buffer (10 mM, pH 7.5) was used for kinetic analysis. 35 μL of PctV variant containing PLP was added to 15 μL of mixture of L-glutamate and 3-DSA, and then incubated at 28 °C for 30 min. Reaction mixtures contained 0.1 μM of PctV variant, 100 μM of PLP, 200 mM of L-glutamate, and various concentration of 3-DSA (1.25, 2.5, 5.0, 6.25, 12.5, 25.0, 50.0, 75.0, 100, 150, and 250 μM). Three mixtures were prepared for each concentration of 3-DSA. Reaction mixtures were quenched by adding 50 μL methanol and centrifuged (15,000 rpm, 30 min). Supernatants of these samples were evaporated and then dissolved in 10 μL of MilliQ-water. These solutions were then injected into an HPLC instrument described above. The elution conditions were 0.1% trifluoroacetatic acid-7.5% CH_3CN-H_2O at a flow rate 0.5 mL/min at room temperature. Obtained data were fitted to Michaelis-Menten equation to determine their kinetic parameters.

Calculation of UV/Vis Absorption. Gaussian 09 program package (Gaussian, Connecticut, USA) was used for optimization of structures and frequency calculation with the B3LYP method and the 6-31G$^+$ basis set. The solvation effect of water was considered by B3LYP/6-31G$^+$.

Stopped Flow Analysis. Twenty five μL of mixture A containing 50 mM of Tris (pH 7.5), 10% glycerol, 100 μM of PctV wild type, and 100 μM of PMP and 25 μL of mixture B containing 50 mM of Tris (pH 7.5), 10% glycerol, and 400 μM of 3-DSA were mixed within 10 ms, and then UV/Vis spectra were measured every 50 ms (total 15 s). Temperature of reaction mixture was kept at 28 ± 0.5 °C.

Preparation of PctV-K276R Mutant for Crystallization. PctV-K276R mutant was expressed and purified by the same method as wild type and then concentrated by Amicon® ultra-4 (Millipore, Darmstadt, Germany). PctV-K276R (150 μM) was reacted with 200 μM of PMP and 1.5 mM of 3-DSA in Tris (50 mM, pH 7.5) buffer containing 200 mM of L-glutamate and 10% glycerol (28°C, 24 hr, 600 μL). The reaction mixture (89.2 μL) was treated with 10 μL of 10 × buffer containing 0.8 μL of 0.2 unit/μL of thrombin (Merck, Darmstadt, Germany) for thrombin cleavage and the mixture was incubated at 28 °C for 3.5 hr. Then streptavidin agarose was added to remove thrombin. After removing of streptavidin agarose, the solution was poured into column with His60 Ni superflow resin (Takara, Shiga, Japan) and the unbounded fractions were used for gel filtration (Superdex™ 200 10/300GL, GE Healthcare UK, Buckinghamshire, England; Tris (50 mM, pH 7.5) buffer containing 5% glycerol and 150 mM of KCl). Fractions containing PctV-K276R were concentrated and then its buffer was replaced with Tris (50 mM, pH 7.5) buffer containing 10% glycerol.

Crystal Structural Analysis. Crystals of native PctV wild type were grown from a 1:1 mixture of a protein solution (4.0 mg/mL in 10 mM Tris-HCl (pH 7.5) and 10% glycerol) and a reservoir solution (0.2 M magnesium chloride, 20% polyethylene glycol 4000, and 0.1 M Tris-HCl (pH 8.5)) using the sitting-drop vapor-diffusion method at 26 °C. Crystals of PctV K276R-PMP-3-DSA complex were grown from a 1:1 mixture of a protein solution (7.5 mg/mL in 50 mM Tris-HCl (pH 7.5) and 10% glycerol) and a reservoir solution (0.2 M magnesium chloride, 24% polyethylene glycol 1000 and MES-NaOH (pH 5.8)) using the sitting-drop vapor-diffusion method at 5 °C. Before X-ray data collection, the crystal was transferred into the reservoir solution supplemented with 25%(v/v) polyethylene glycol 400 or 10% (v/v) glycerol as a cryoprotectant and flashfrozen in the liquid nitrogen stream. X-ray data were collected using beamline AR-NW12A (Photon Factory, Tsukuba, Japan). All diffraction data were indexed, integrated, and scaled using the program iMosflm.[15] The initial phases were determined by the molecular replacement method using the program Molrep[16] with the crystal structure of GSAM (Protein Data Bank Code: 3FQ7). The program ARP/wARP[17] was used for automatic initial protein model building and refinement. Coot[18] was used for visual inspection and manual rebuilding of the model. Refmac[19] was used for refinement. The figures were prepared using PyMOL (The PyMOL Molecular Graphics System, DeLano Scientific LLC, Palo Alto, CA.). The geometries of the final structures were evaluated using the program Rampage.[20] The resulting coordinates and structure factors

have been deposited in the Protein Data Bank (PDB codes: 4ZM3 and 4ZM4).

UV/Vis Spectral Analyses. To investigate whether K276Q mutant accumulates any intermediate, UV/Vis spectra analyses were performed. 3 μL of 3-DSA was added to 147 μL of mixture containing 10 mM Tris (pH 7.5), 10% glycerol, PctV (wild type or K276Q) and PMP. Reaction mixture contained 50 μM of PctV, 50 μM of PMP and 200 μM of 3-DSA. UV/Vis spectra were measured every 60 sec (total 1 hr) at room temperature. UV/Vis spectra of mixture without enzyme were also measured.

To investigate whether K276R mutant accumulates any intermediate, 6 μL of 3-DSA was added to 144 μL of mixture containing 10 mM Tris (pH 7.5), 10% glycerol, 200 mM L-glutamate, K276R variant, and PMP, and then UV/Vis spectra were measured every 30 min (total 12 hr). Reaction mixture contained 40 μM of PctV, 40 μM of PMP and 400 μM of 3-DSA.

To investigate PMP forming ability of S28A and R31A mutants, 15 μL of L-glutamate was added to 135 μL of the mixture containing 10 mM Tris (pH 7.5), 10% glycerol, PLP, and PctV (wild type, S28A, or R31A), and then UV/Vis spectra were measured every 60 sec (total 10 min). Reaction mixture contained 7.5 μM of PctV, 60 μM of PLP and 1, 20, or 200 mM of L-glutamate.

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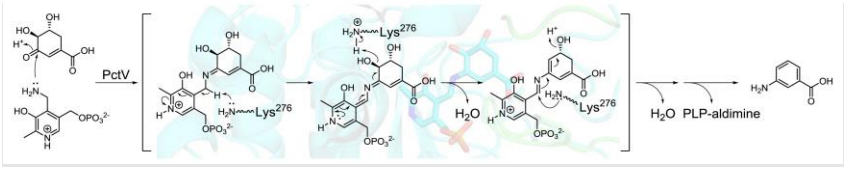

Purple quinonoid intermediate of pyridoxamine 5'-phosphate and 3-dehydroshikimate was accumulated in the K276R mutant of 3-aminobenzoate synthase PctV in the biosynthesis of pactamycin. X-ray protein structural analysis of the complex revealed the quinonoid molecule trapped at the active site of the mutant. Overall, the active site Lys found to have a multiple role including the aminotransfer reaction and dehydration to give 3-aminobenzoate.

Akane Hirayama, Akimasa Miyanaga, Fumitaka Kudo, and Tadashi Eguchi**

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Mechanism-based Trapping of the Quinonoid Intermediate Using the K276R Mutant of PLP-Dependent 3-Aminobenzoate Synthase PctV in the Biosynthesis of Pactamycin