Pyridoxal-5′-phosphate as an oxygenase cofactor: Discovery of a carboxamide-forming, α-amino acid monoxygenase-decarboxylase

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Capuramycins are antituberculosis antibiotics that consist of a modified nucleoside named uridine-5′-carboxamide (CarU). Previous biochemical studies have revealed that CarU is derived from UMP, which is first converted to uridine-5′-aldehyde in a reaction catalyzed by the dioxygenase CapA and subsequently to 5′-C-glycyluridine (GlyU), an unusual β-hydroxy-α-amino acid, in a reaction catalyzed by the pyridoxal-5′-phosphate (PLP)-dependent transaldolase CapH. The remaining steps that are necessary to furnish CarU include decarboxylation, O atom insertion, and oxidation. We demonstrate that Cap15, which has sequence similarity to proteins annotated as bacterial, PLP-dependent α-oxo-IRNA (Sec) selenium transferases, is the sole catalyst responsible for complete conversion of GlyU to CarU. Using a complementary panel of in vitro assays, Cap15 is shown to be dependent upon substrates O2 and (5′,S,6′)-GlyU, the latter of which was unexpected given that (5′,S,6′)-GlyU is the isomeric product of the transaldolase CapH. The two products of Cap15 are identified as the carboxamide-containing CarU and CO2. While known enzymes that catalyze this type of chemistry, namely α-amino acid 2-monoxygenase, utilize flavin adenine dinucleotide as the redox cofactor, Cap15 remarkably requires only PLP. Furthermore, Cap15 does not produce hydrogen peroxide and is shown to directly incorporate a single O atom from O2 into the product CarU and thus is an authentic PLP-dependent monoxygenase. In addition to these unusual discoveries, Cap15 activity is revealed to be dependent upon the inclusion of phosphate. The biochemical characteristics along with initiatory mechanistic studies of Cap15 are reported, which has allowed us to assign Cap15 as a PLP-dependent (5′,S,6′)-GlyU: O2 monoxygenase-decarboxylase.

Significance

Enzymes that activate dioxygen typically rely on flavins or transition metals as redox cofactors. We describe the discovery of the enzyme catalyst Cap15 that converts 5′-C-glycyluridine (GlyU), an unusual β-hydroxy-α-amino acid, to the modified nucleoside uridine-5′-carboxamide. In contrast to expectations, pyridoxal-5′-phosphate (PLP) is the sole cofactor that serves as the initiating reducing agent to activate dioxygen for incorporation into GlyU prior to decarboxylation. Thus, Cap15 is now classified as an O2- and PLP-dependent monoxygenase-decarboxylase. Also in contrast to expectations, phosphate and the (5′,S,6′) isomer of GlyU—and not (5′,S,6′)-GlyU, the established pathway intermediate—are essential for Cap15 activity. In total, Cap15 utilizes an enzymatic strategy for oxygen incorporation during conversion of an α-amino acid to a carboxamide.


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Bioinformatic Analysis of Cap15. The gene product of cap15 has moderate sequence identity (45–50%) to proteins annotated as putative t-secyl-tRNA(Sec) selenium transferase, SelA. Secondary-structure-based predictions mirrored this analysis with the highest similarity to Aquifex aeolicus SelA (13), although sequence identity by pairwise alignment was significantly lower (24%, SI Appendix, Fig. S1A). Bacterial SelA catalyzes the PLP-dependent production of selenocysteinyl-tRNA(Sec) and selenophosphate from substrates t-secyl-tRNA(Sec) and selenophosphate (SI Appendix, Fig. S2). Studies with AuSelA have revealed that this enzyme functions as a pentamer of dimers, with residues from each dimer subunit (termed A and B subunits) contributing to catalysis (13), and the reaction is initiated by formation of an internal aldime between Lys265 and PLP. Based on sequence comparison, Lys265 is predicted to be the respective residue in Cap15 (SI Appendix, Fig. S1A). Only two additional Lys residues are conserved (Lys139 and Lys362 of AuSelA corresponding to Lys265 and Lys303 of Cap15, respectively). As part of the B subunit, the former Lys is located within ∼12 Å of the PLP and selenophosphate binding site of the A subunit; in contrast, the latter Lys is located distant from the active site. The importance of either residue has not been established. The B subunit of the AuSelA dimer also has two essential Arg residues that are proposed to establish an important electrostatic interaction for binding of PLP and the substrate selenophosphate (SI Appendix, Fig. S1B). While one of the Arg residues is conserved in Cap15 (Arg286), the second is substituted with a Lys (Lys303) (SI Appendix, Fig. S1A).

Functional Assignment of Cap15. Recombinant His6-Cap15 was soluble upon production in Escherichia coli and purified to near homogeneity using immobilized metal affinity chromatography (SI Appendix, Fig. S3A). Cap15(K230A) was also prepared as a potential control (SI Appendix, Fig. S3A). Both purified protein solutions were colorless and lacked a UV/Vis spectrum indicative of a PLP cofactor. The hypothetical substrate 5′-6′GlyU-GlyU, which was identified as the stereoisomer produced by the CapH-homolog LipK (47% sequence identity via pairwise alignment) that is involved in the biosynthesis of A-90289 (Fig. 1) (14), was synthesized (SI Appendix, SI Materials and Methods and Fig. S4) and tested with Cap15 in Heps buffer with PLP. Following overnight reactions, HPLC analysis did not reveal any new peaks compared with the controls. In contrast to LipK (14), the stereochemistry of the CapH product was not previously assigned (11). Therefore, two additional stereoisomers of GlyU—(5′,6′R,S′)-GlyU and (5′,6′R,S′)—that could hypothetically arise from the transaldolase reaction (14) were tested for activity with Cap15. We have detailed the stereocontrolled synthesis and analytic characterization of these two diastereomers elsewhere (15–17). Once again, however, no new peaks were observed with Cap15.

We next explored a variety of reaction conditions including different pH and buffer systems. When reactions were performed in phosphate buffer with the supposedly unnatural (5′,6′R,S′)-GlyU isomer, a new peak was observed by HPLC, and formation of the new peak was dependent upon the inclusion of Cap15 and PLP (Fig. 2A). A trace amount of the new peak appeared following reactions with Cap15(K230A) (Fig. 2A). No new peaks were detected using the (5′,6′S,R′) or (5′,6′R,S′) isomers of GlyU as potential substrates under the phosphate-buffered conditions (SI Appendix, Fig. S5). The new peak was analyzed by LC-MS yielding an (M+H)+ ion at m/z = 288.0, unexpectedly consistent with the molecular formula for CarU [expected (M+H)+ ion at m/z = 288.1 and (M+Na)+ ion at m/z = 310.0].
To simplify product identification, CarU was synthesized and purified, and authentic CarU coeluted with the Cap15 product and had identical mass and NMR spectroscopic properties (Fig. 2A and SI Appendix, Figs. S6–S11). HPLC analysis of the Cap15 reaction revealed the enzyme was inactive under anaerobic conditions (Fig. 2A) and inclusion of EDTA had no effect (SI Appendix, Fig. S12). The specific activity with Cap15(K230A) was (2.4 ± 0.1) × 10⁻⁴ μmol/min/mg under the optimized conditions. The specific activity with Cap15(K230A) was (2.4 ± 0.1) × 10⁻⁴ μmol/min/mg, an approximately 20-fold decrease compared with the wild-type enzyme. Using HPLC to detect CarU formation, single-substrate kinetic analysis with variable (5′S,6′R)-GlyU revealed Michaelis–Menten kinetics yielding a $K_m$ = (56 ± 7) × 10⁻⁵ μM and $k_{cat}$ = (9.3 ± 0.5) × 10⁻³ min⁻¹ (Fig. 3B). Increasing the concentration of phosphate from 50 mM to 1 M had little impact with respect to the overall catalytic efficiency [$k_{cat}$ = (42 ± 4) × 10⁻³ μmol/min/mg and $k_{cat}$ = (6.2 ± 0.2) × 10⁻₃ min⁻¹] (SI Appendix, Fig. S13). Furthermore, nearly identical kinetic parameters were calculated when activity was measured by CO₂ detection instead of CarU detection by HPLC (Fig. 3B).

As previously noted, the activity of Cap15 was stereoselective for the (5′S,6′R)-GlyU diastereomer. Thus, the reaction catalyzed by CapH was re-examined to identify the stereochemistry of the GlyU product. Initially, a one-pot reaction with CapH and Cap15 starting from U5′S and 1-Thr was analyzed by HPLC, revealing the formation of a peak coeluting with GlyU (SI Appendix, Fig. S14). However, no CarU was observed, suggesting that the CapH product—presumably (5′S,6′S)-GlyU—was not a substrate for Cap15. Subsequently, the stereochemistry of the CapH product was directly assessed. As described elsewhere (14), phosgene modification of the β-hydroxy-amino acid of GlyU enabled the analytical separation of the three synthetic diastereomers in hand. Following the CapH reaction using synthetic U5′S and 1-Thr as substrates, the phosgene-modified GlyU coeluted with authentic, phosgene-modified (5′S,6′S)-GlyU (Fig. 3C). Modification of the LipK product as a control confirmed this result (14). Thus, CapH stereospecifically generates (5′S,6′S)-GlyU, which must undergo epimerization before the Cap15-catalyzed reaction.

Initiator Mechanistic Analysis. The K230A mutation of Cap15 did not abolish activity as initially predicted, and thus site-directed mutants of the two remaining conserved Lys residues (Lys⁶⁰⁶ and Lys⁶⁰⁹) were prepared, and the mutant proteins analyzed for activity. Cap15-Lys⁶⁰⁶ was also targeted for mutagenesis due to...

Fig. 2. Biochemical characterization of Cap15. (A) HPLC traces of the reaction catalyzed by Cap15 using substrate (5′S,6′R)-GlyU with (i) exclusion of enzyme; (ii) complete reaction mixture containing phosphate, (5′S,6′R)-GlyU, O₂, PLP, and Cap15; (iii) exclusion of PLP; (iv) substitution of Cap15(K230A) for the wild-type enzyme; (v) exclusion of O₂; and (vi) synthetic CarU. Avas, absorbance at 260 nm. (B) LC-MS analysis of the CarU product following Cap15-catalyzed reactions in the presence of the indicated isotopically labeled molecule. Data shown are representative of duplicates, RA, relative abundance.
the importance of the corresponding Arg residue in AaSelA (SI Appendix, Fig. S1). The three additional Lys → Ala mutant proteins were soluble when expressed in E. coli (SI Appendix, Fig. S3A), and activity analysis by HPLC revealed that Cap15(K303A) retained activity comparable to the wild-type enzyme (SI Appendix, Fig. S15). Contrastingly, the K262A and K265A mutant proteins did not produce detectable levels of CarU. The UV/Vis absorption spectra for the wild-type and mutant proteins were examined with the goal of establishing a mechanistic framework for Cap15 (2, 18, 19). Wild-type Cap15 and the mutant variants did not have an obvious absorbance at wavelengths greater than 300 nm, consistent with the production of the recombinant enzyme in an apo-form (Fig. 4A, trace I, and SI Appendix, Fig. S16A). The lone exception was Cap15(K265A), which displayed a λmax = 411 nm that is consistent with some protein copurifying with PLP as the internal aldimine (SI Appendix, Fig. S16A). Identical results were obtained when including excess PLP before protein purification by affinity chromatography. Following the addition of substoichiometric PLP, which has a λmax at 388 nm representing the aldehyde form (Fig. 4A, trace II), a subtle red shift to λmax at 391 nm was detected for wild-type Cap15 (Fig. 4A, trace III), Cap15(K262A), and Cap15(K303A) (SI Appendix, Fig. S16B); the spectrum for Cap15(K230A) was essentially identical to free PLP. The λmax = 411 nm for Cap15(K265A) did not shift but increased in intensity following the addition of PLP (SI Appendix, Fig. S16B). Upon addition of excess (5′S,6′R)-GlyU to the wild-type Cap15-PLP solution, a red shift was observed to λmax = 415 nm (Fig. 4A, trace IV). Cap15(K230A) and Cap15(K303A), both active as catalysts, had similar spectral shifts (SI Appendix, Fig. S16C). In contrast, the UV/Vis spectra for Cap15(K262A) and Cap15(K265A) did not change (SI Appendix, Fig. S16C). As expected, nonsubstrates (5′S,6′S)-GlyU and (5′R,6′S)-GlyU did not significantly alter the spectral profile when added to the wild-type Cap15-PLP mixture (SI Appendix, Fig. S17). Closer examination of the Cap15 UV/Vis spectrum revealed a second λmax at 503 nm, which appeared immediately following the addition of (5′S,6′R)-GlyU and remained nearly constant over the course of 24 min (Fig. 4B, trace V). In contrast to the other mutant proteins, only Cap15(K303A) displayed the identical λmax at 503 nm (SI Appendix, Fig. S16D).

**Discussion**

The functional assignment of Cap15 has revealed unprecedented enzyme chemistry that involves PLP as an oxygenase cofactor, an unusual role for an already well-known and highly versatile cofactor. Several enzymes involved in primary metabolism that...
generate carbanion intermediates have previously been reported to catalyze off-pathway side reactions—so-called paracatalytic reactions—that involve oxidation of the carbanionic intermediate with O_2 (20, 21). Given that most members of the PLP-dependent transferase superfamily are characterized by their ability to stabilize a carbanion through electron delocalization within PLP, it is not surprising that five distinct PLP-dependent enzymes are included in the paracatalytic reaction list (20). These five PLP-dependent enzymes use an amino acid substrate to catalyze oxidative deamination in addition to decarboxylation, the former of which was not detected with Cap15. For three of these PLP-dependent enzyme “paracatalysts,” H_2O_2 is stoichiometrically produced, and thus they are classified as oxidases (20, 22–25). The fate of the oxygen atoms for the other two paracatalysts, however, remains unknown.

In contrast to PLP-dependent enzymes that catalyze paracatalytic reactions, a few enzymes have recently been reported to catalyze PLP-dependent oxidations as their primary function (3, 4, 26, 27). Ind4, involved in indolylmec biosynthesis, was shown to catalyze tandem 2,3- and 4,5-dehydrogenation of α-Arg, concomitantly reducing O_2 to H_2O_2 (3). CcdF, involved in celesticin biosynthesis, was shown to catalyze an oxidative decarboxylation and deamination of an α-amino-acid component of a celesticin precursor, also concomitantly reducing O_2 to H_2O_2 (26). Thus, both Ind4 and CcdF are bona fide PLP-dependent oxidases. Contrastingly, MppP involved in 1-enduracidine biosynthesis was shown to catalyze an O_2 and PLP-dependent α-deamination and 4-hydroxylation of α-Arg, the latter suggesting that this enzyme is an oxygenase (4). However, neither the fate of the oxygen atoms of O_2 nor the origin of the oxygen atom in the hydroxylated product was determined, and thus the enzyme classification of MppP is not clear. Our results definitively establish the use of PLP as a redox cofactor that enables Cap15 to function as a true monooxygenase, incorporating one O atom of O_2 directly into the carboxamide-containing product CarU. Interestingly, a similar PLP-dependent, α-amino-acid-to-carboxamide transformation has been proposed during pyoverdine biosynthesis, although the activity of the probable catalyst (PvDN) remains to be demonstrated in vitro (27).

Bioinformatically, Cap15 has primary sequence homology to bacterial proteins annotated as SeLa, which is a fold-type I PLP-dependent enzyme that catalyzes the transformation of seryl-tRNA (Ser) to seryl-tRNA (Ser) with the selenocysteinyl residue as the selenium cofactor (13). The discovery that the Cap15 reaction not only requires O_2 but also depends on phosphate, in this case the standard oxygenated variant, gave rise to the possibility that the O atom incorporated into CarU could originate directly from phosphate in a reaction analogous to that catalyzed by SeLa. However, the isotopic enrichment studies with 18O-labeled phosphate, compared with 16O-labeled O_2, clearly revealed that this was not the case, thus leaving the role of phosphate in the Cap15-catalyzed reaction unknown. Interestingly, the dioxygenase CapA, which initiates the CarU biosynthetic pathway, produces phosphate via an unusual oxidative dephosphorylation mechanism that could potentially participate as a feed-forward allosteric modulator (11). Preliminary analysis suggests that Cap15 does in fact differentially oligomerize in the presence of phosphate (SI Appendix, Fig. S1B), although ongoing kinetic and structural studies will be essential to clarify the quaternary structure and role of phosphate.

Wild-type Cap15 (1) does not copurify with a detectable amount of PLP (Fig. 4B) and is inactive without an exogenous supply of PLP. Furthermore, the addition of PLP to Cap15 (III) resulted in a UV/Vis spectrum that is nearly indistinguishable from the aldheyde form of PLP (II), which is in contrast to most other PLP-dependent enzymes that display a significant red shift (λ_max of 410–440 nm) that is indicative of an internal aldmine (19). As a result, we initially considered a mechanism that bypasses the internal aldmine. However, studies with PLP-dependent aspartate aminotransferase (28) and 1-aminocyclopropane-1-carboxylic synthase (29), among others (2, 19), have revealed an internal aldmine that—depending upon the orientation and protonation state of PLP in the active site—can display a UV/Vis spectrum with a λ_max ranging from 390 nm (corresponding to an unprotonated imine) to 420 nm (corresponding to a protonated imine). Additionally, mutational analysis of Cap15 revealed that the inactive mutant Cap15(K265A) displays a spectrum with a λ_max of 411 nm following purification and addition of exogenous PLP, which is consistent with formation of a protonated internal aldmine (30, 31). In lieu of the literature precedent along with these data, we propose a mechanism wherein Cap15 does indeed form a protonated internal aldmine (III) with an unidentified Lys that is characterized by a λ_max of 391 nm (Fig. 4B). Mutation of Lys260 is proposed to promote orientation of the internal aldmine to favor protonation, hence displaying the more traditional spectrum associated with PLP-dependent enzymes. Regardless, the red shift to λ_max at 416 nm following the addition of (S,S,6,R)-GlyU to the Cap15-PLP complex strongly suggests the formation of an external aldmine (IV) that undergoes Co-deprotonation to form a resonance-stabilized quinonoid (V), which is characterized by the appearance of a second shoulder at 503 nm in the spectrum for wild-type Cap15 and Cap15(K303A). Similar to the results with MppP and Ind4 (3, 4), the quininoid appears to reach a steady state during the reaction that would suggest that downstream chemistry is rate-limiting (2).

The most intriguing aspect of the Cap15 reaction is the oxidative steps that follow aldmine formation. Other enzymes that catalyze oxidative decarboxylation of α-amino acids to form carboxamides—namely Trp (32), Lys (33), Arg (34), and Phe (35) 2-monoxygenases—require flavin adenine dinucleotide (FAD) to initiate electron transfer to dioxygen (36). For Trp-2-monoxygenase, the best characterized of the carboxamide-forming flavoproteins, the reaction begins by hydride transfer from Trp to FAD to generate an imine and reduced FAD, respectively (32). The reduced FAD subsequently donates an electron to O_2 to yield a caged radical pair that, following spin inversion, recombines to form a C(4a)-hydroperoxide intermediate (34). Contrastingly, the oxidative steps of the Cap15-catalyzed reaction do not require a flavin cofactor, nor does it appear that a redox active metal cofactor is involved. Consequently, we propose a resonance-stabilized quininoid intermediate (Cap15 (IV) wherein the carbanion resonance form of the quininoid (V) serves a role that is comparable to the reduced FAD for Trp-2-monoxygenase. Thus, an electron is first transferred from the carbonization to O_2 to form a resonance-stabilized GlyU-PLP radical and superoxide, the latter of which can undergo protonation and rebound with the carbon-centered radical to form the hydroperoxide species. Subsequently, decarboxylation occurs with elimination of the distal hydroperoxyl O atom in the form of water to generate a CarU-PLP aldmine that is ultimately hydrolyzed to release CarU. Although it remains possible that the initial carbonization is generated by decarboxylation instead of deprotonation of the aldmine, we were unable to detect CO_2 under anaerobic conditions, thus suggesting that O_2 reduction precedes decarboxylation.

In addition to the unusual role of PLP as a redox cofactor, the finding that Cap15 is stereospecific for the (5′S,6′R)-disteromer of GlyU was unexpected. This led us to reexamine the stereochemical outcome of the transaldolase CapH. Although it is mechanistically feasible for CapH to produce (5′S,6′R)-GlyU, the product was clearly identified as (5′S,6′R)-GlyU upon comparisons with synthetic standards and the product of the well-characterized transaldolase LipK (14). Thus, the discovery of Cap15 activity—despite filling a major gap regarding the biosynthetic mechanism of CarU—introduces another biochemical step in the pathway and suggests the involvement of an additional, unidentified epimerase. Intriguingly, a putative NAD-dependent
NDP-hexose epimerase is among the few remaining unassigned proteins within the gene cluster; however, other possibilities cannot be excluded at this time.

In summary, we have provided evidence to support the functional assignment of Cap15 as a PLP-dependent (5′,3′,6′R)-GlyU: O₂ monooxygenase-decarboxylase that generates the carboxamide 32:8092 16590. sp. P-501.

Materials and Methods

Cloning, mutagenesis, heterologous production, and activity analysis for Cap15 were performed using standard procedures and are described in the SI Appendix: SI Materials and Methods. Reactions were monitored by C18 reverse phase HPLC, LC-MS, a Carbon Dioxide Enzymatic Assay kit (Diazyme Laboratories), and a Profiling Oxygen Microsensor PM-PS7 equipped with Microx 4 oxygen meter (PreSens Precision Sensing). UV-visible spectroscopy was performed with a Shimadzu UV/Vis-1800 spectrophotometer. The synthesis of (5′R,6′S)-GlyU, (5′,6′R)-GlyU, and US A was previously described (14–17). The syntheses and analytical analyses of (5′,6′S)-GlyU and CarU are described in the SI Appendix: SI Materials and Methods.

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