Formation of an aminoacyl-*S*-enzyme intermediate is a key step in the biosynthesis of chloramphenicol†

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Herein we report the first biochemical characterization of an enzyme involved in the biosynthesis of chloramphenicol that provides new insights into the origins of the antibiotic.

Chloramphenicol, produced by *Streptomyces venezuelae*, is a potent antibiotic whose mechanism of action is inhibition of protein synthesis *via* binding to the 50S subunit of the bacterial ribosome. Its unusual structure, which includes a rarely observed aromatic nitro group and a dichloroacetamide moiety, has motivated many investigations into the antibiotic's biosynthesis.[1] Precursor labelling experiments proved that L-*para*-aminophenylalanine, a non-proteinogenic amino acid, is a direct precursor of chloramphenicol[1] (Fig. 1A). Accordingly, the chloramphenicol biosynthesis pathway is known to consist of the synthesis, β-hydroxylation, *N*-dichloroacetylation, *N*-oxygenation, and reduction of *p*-aminophenylalanine.

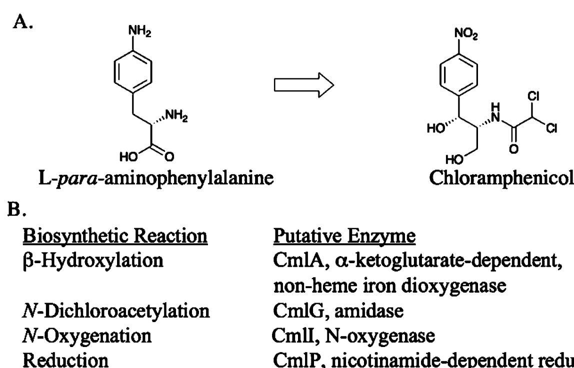

Fig. 1 (A) Chemical structures of chloramphenicol and its precursor, L-para-aminophenylalanine. (B) The reactions and their putative catalysts for the conversion of L-*p*-aminophenylalanine into chloramphenicol.

The presumed enzymes for these reactions were identified by bioinformatic analysis of the open-reading frames identified in the sequence of the genetic locus for chloramphenicol biosynthesis in *S. venezuelae*[2] (Fig. 1B). Thus far, genetic analyses of the biosynthetic locus in *S. venezuleae* have not validated the proposed functions of the enzymes encoded by these genes or revealed the order of the reactions in the biosynthesis.[1,2] Herein we report the first biochemical characterization of an enzyme implicated in the biosynthesis of chloramphenicol.

The subject of this study is the enzyme coded by the *cmlP* gene. CmlP shows sequence similarity and protein domain organization with the yeast α-aminoadipate reductase (Lys2) involved in L-lysine biosynthesis.[3] Both enzymes contain an N-terminal adenylation domain, an internal peptidyl carrier domain, and a C-terminal domain that is homologous to nicotinamide-dependent dehydrogenases. Analysis of Lys2 established that the first two domains are involved in α-aminoadipate recognition followed by autoaminoacylation to generate an aminoacylthioester intermediate. The C-terminal domain subsequently catalyzes the reductive release of this intermediate resulting in α-aminoadipate semialdehyde formation.[3] Based on this precedent, the catalytic strategy of CmlP could be an iterative cycle of autoaminoacylation of the constituent phosphopantetheine moiety and reduction of the resulting aminoacyl thioester. A set of experiments was designed and performed to test this hypothesis.¶

CmlP, like Lys2 and the non-ribosomal synthetases, has an adenylation domain. These domains catalyze the ATP-dependent conversion of amino acids into reactive aminoacyl adenylates, which is required for autoaminoacylation. Because L-*p*-aminophenylalanine is a known precursor of chloramphenicol, we hypothesized that it would be the favored co-substrate of the CmlP adenylation domain. To enable assays of its activity, CmlP was heterologously over-produced in *E. coli* and purified to homogeneity by sequential Ni^{2+}-chelate and ion-exchange chromatography. By convention,[4] adenylation of L-*p*-aminophenylalanine by CmlP was assessed indirectly by measuring the rate at which *apo*-CmlP catalyzed exchange of the radiolabel from [^{32}P]-pyrophosphate to ATP in the presence of the amino acid. The kinetic parameters of the exchange reaction were determined ($K_m = 0.90 \pm 0.01$ mM, $k_{cat} = 7.3 \pm 0.2$ min^{-1}) and used to calculate a catalytic efficiency (k_{cat}/K_m) of 8.11 mM^{-1} min^{-1}. These parameters are very similar to those reported for a homologous adenylation domain in the presence of its cognate amino acid co-substrate.[5] There is a strong preference for L-*p*-aminophenylalanine, as CmlP catalyzed pyrophosphate exchange at markedly lower rates in the presence of the proteinogenic, aromatic amino acids—tyrosine and phenylalanine (Table 1). Interestingly, CmlP-catalyzed pyrophosphate exchange was nearly undetectable when L-*p*-aminophenylalanine was replaced with either L-*p*-nitrophenylalanine or racemic *threo*-phenylserine. These results have implications for the order of reactions in chloramphenicol biosynthesis. The low degree to which L-*p*-nitrophenylalanine stimulates the catalysis of pyrophosphate

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Table 1 Kinetic parameters of the CmlP-catalyzed pyrophosphate exchange in [^{32}P]PPi–ATP assays with L-*p*-aminophenylalanine (L-*p*-APA), L-phenylalanine (L-Phe), and L-tyrosine (L-Tyr)

Amino acid	K_m/mM	k_{cat}/min^{-1}	k_{cat}/K_m/mM^{-1}min^{-1}
L-*p*-APA	0.90 ± 0.01	7.3 ± 0.2	8.11
L-Phe	9.40 ± 0.90	0.90 ± 0.03	0.096
L-Tyr	4.10 ± 0.43	1.97 ± 0.11	0.48

exchange rules out the possibility that *N*-oxygenation of L-*p*-aminophenylalanine precedes its adenylation in the biosynthesis of chloramphenicol. Likewise, the adenylation is likely to precede β-hydroxylation in the biosynthetic sequence since phenylalanine stimulates CmlP catalysis of pyrophosphate exchange while *threo*-phenylserine, the β-hydroxylated form of phenylalanine, does not. In total, these data suggest that the CmlP-mediated adenylation of L-*p*-aminophenylalanine is the first step in its conversion to chloramphenicol.

By analogy to the mechanism of Lys2,[3] we proposed that CmlP activates and attaches L-*p*-aminophenylalanine to itself *via* a thioester linkage to the phosphopantheteine moiety bound to its peptidyl carrier domain. To assay autoaminoacylation, *holo*-CmlP was incubated with [^{3}H]-L-*p*-aminophenylalanine and ATP and the enzyme's incorporation of the radiolabel was measured by liquid scintillation counting. In this experiment, nearly 50% of the *holo*-CmlP is autoaminoacylated within 50 minutes (Fig. 2). The rate and degree of *p*-aminophenylalanyl-*S*-CmlP formation are consistent with those reported for related enzymes.[3,5]

Based on the mechanism of Lys2 and related enzymes,[3,6,7] the reductase domain of CmlP is expected to reduce an aminoacyl-*S*-enzyme intermediate. Using loss of radiolabel as an indicator of reductive release, the activity of the CmlP reductase domain was assessed by measuring the stability of [^{3}H]-L-*p*-aminophenylalanyl-*S*-CmlP in the presence of nicotinamide co-factors. Using cold

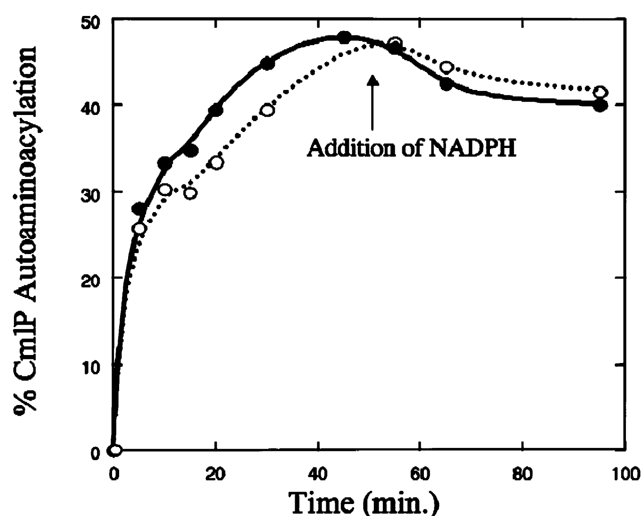
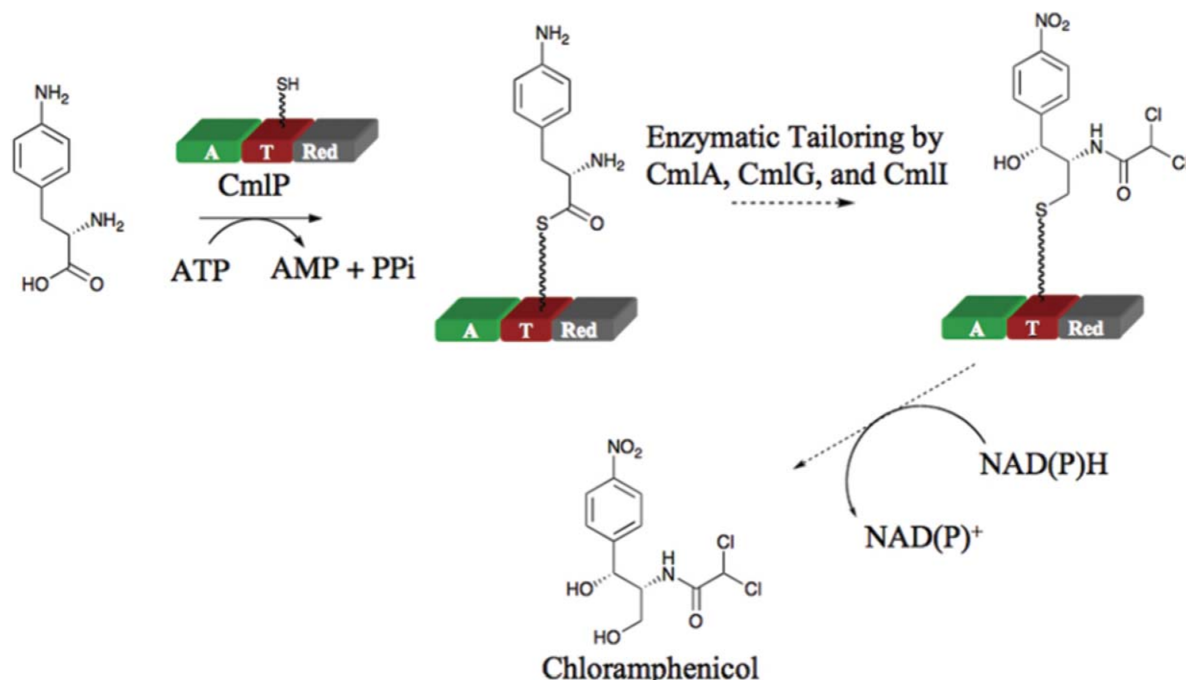


Fig. 2 Time course of CmlP autoaminoacylation with [^{3}H]-L-*p*-aminophenylalanine (in black, closed circles) and responses of the L-*p*-aminophenylalanyl-*S*-enzyme intermediate to the addition of NADPH to a concentration of 10 mM (dotted line, open circles). L-*p*-Aminophenylalanine was added as a cold chase.

p-aminophenylalanine as a chase, this intermediate was found to be stable in the presence of NADPH (Fig. 2) and NADH (data not shown). As there are no indications that the reductase domain is improperly folded or otherwise inactive, the most plausible explanation of this result is that CmlP-bound *p*-aminophenylalanine is not the substrate of the reductase domain and that the amino acid is enzymatically modified at least once before it is reductively released. In fact, there are precedents for the β-hydroxylation and *N*-acylation of enzyme-bound amino acids in antibiotic synthetic pathways by functional homologs of CmlA and CmlG, respectively.[5,8]



Scheme 1 Proposal for the biosynthesis of chloramphenicol.

Our biochemical analysis of CmlP has provided the first insights into the molecular logic of the chloramphenicol biosynthesis. The conclusion drawn from our study is that CmlP autoaminoacylation with L-*p*-aminophenylalanine is the first committed step in its conversion to chloramphenicol. The results also suggest that through this reaction, the amino acid is primed not only for reduction, but at least one of the other three enzymatic transformations in the biosynthetic pathway. Based on bioinformatic analyses and literature precedents,[2,5] we propose that CmlG and CmlA catalyze *N*-dichloroacetylation and β-hydroxylation of the aminoacyl-*S*-enzyme intermediate. After these tailoring reactions and perhaps *N*-oxygenation by CmlI, the amino acid would be reductively released *via* the action of the CmlP reductase domain (Scheme 1). This unusual catalytic strategy of tailoring an aminoacyl-*S*-enzyme intermediate by enzymes acting *in cis* and *in trans* is another example on the growing list of variations on the assembly-line enzymology strategy for antibiotic biosynthesis.[9] Validation of this model will require reconstitution of the activities of the other chloramphenicol biosynthetic enzymes, which has so far been not been achieved.

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Notes and references

¶ **Heterologous overproduction and purification of CmlP.** Details concerning the cloning of *cmlP*, heterologous overproduction of CmlP in *E. coli*, and purification of CmlP are provided in the ESI.† **[^{32}P]PPi–ATP exchange assays.** Reactions were carried out under standard conditions. Reactions for amino acid specificity contained 2 μM and 1 mM of L-*p*-aminophenylalanine, L-phenylalanine, L-tyrosine, L-*p*-nitrophenylalanine, or racemic phenylserine. For determining the kinetic parameters of pyrophosphate exchange in the presence of L-*p*-aminophenylalanine, the reactions were carried out for 10 minutes with varying concentrations of the amino acid. The reaction time was lengthened to 30 minutes and the enzyme concentration was increased to 4 μM to determine kinetic parameters for pyrophosphate exchange with tyrosine and phenylalanine. Assays were performed in triplicate for each concentration of amino acid. The reactions were in the linear range for enzyme concentration and $<10\%$ substrate-to-product conversion. **Autoaminoacylation assays.** Reactions were carried out in solution containing 75 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM ATP, 1 mM tris-(2-carboxyethyl)phosphine hydrochloride, 1 mM L-*p*-aminophenylalanine, 50 μM [^{3}H] *p*-aminophenylalanine (2 Ci mmol^{-1}, custom synthesized by Moravek Biochemicals, Inc.) with 2 μM of holo CmlP. Samples were quenched by precipitating protein with 10% TCA. After washes with 10% TCA, the protein was resuspended in 88% formic acid and the amount of radiolabel incorporated was measured by liquid scintillation counting. The percent modification of CmlP was calculated from the specific activity of the [^{3}H]-L-*p*-aminophenylalanine and the holo-CmlP concentration. A coomassie-stained SDS-PAGE gel and a corresponding autoradiogram of CmlP incubated with [^{3}H]-L-*p*-aminophenylalanine and with or without ATP are included in the ESI† to show that the radiolabel detected by these assays is associated with CmlP. **Reductase activity assays**. The [^{3}H]-L-*p*-aminophenylalanyl-*S*-CmlP intermediate was formed as described above. After 50 minutes, 1 mM L-*p*-aminophenylalanine was added to the reaction as a cold chase and varying concentrations of NADPH or NADH. The samples were processed and the percent modification of CmlP was calculated as described above.

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