Origin of the β-Lactam Carbons in Clavulanic Acid from an Unusual Thiamine Pyrophosphate-Mediated Reaction

Nusrat Khaleeli, Rongfeng Li, and Craig A. Townsend*

Department of Chemistry, The Johns Hopkins University Baltimore, Maryland 21218

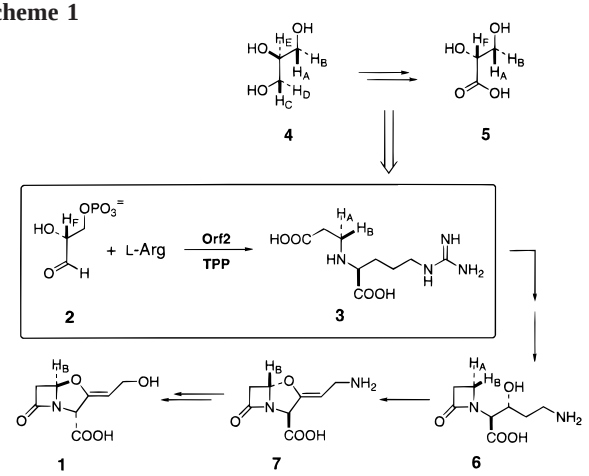
Received July 6, 1999

Clavulanic acid (**1**) is a potent, naturally occuring inhibitor of β-lactamases in bacteria. These enzymes confer resistance to penicillin and cephalosporin by catalyzing the hydrolysis of these antibiotics rendering them inactive.[1,2] The primary metabolic precursors of clavulanic acid are known to be arginine[3,4] and a C_3-intermediate thought to be derived from glycolysis.[5] Identification of the latter has defied assiduous investigation over many years.[6,7] In this paper we report that the first gene of the clavulanic acid gene cluster in *Streptomyces clavuligerus* encodes a thiamine pyrophosphate (TPP)-dependent enzyme that carries out the unprecedented condensation of L-arginine with D-glyceraldehyde-3-phosphate (**2**) to give N^2-(2-carboxyethyl)arginine (**3**, CEA), the first dedicated intermediate in clavulanic acid biosynthesis.[8]

Detailed isotopic labeling experiments have placed strict constraints on the mechanism of the coupling reaction that links the C_3- and C_5-building blocks drawn from primary metabolism to initiate clavulanic acid biosynthesis. It is known that H-2 of both glycerol (**4**, H_E),[6,9] and glyceric acid (**5**, H_F)[7,10] are lost on incorporation into clavulanic acid. Of the four remaining glycerol hydrogens (**4**, H_{A-D}), only one is retained in **1**.[6] The identity of this single hydrogen (H_B) was determined in a stereochemical experiment in which only the *pro-(R)* arm of glycerol was radiolabeled specifically at one or the other diastereotopic methylene locus (**4**, H_A or H_B).[11] This telling result implied that, since stereochemical information is retained through the intermediates of glycolysis as far as phosphoenol pyruvate (PEP), the biosynthetic pathway must proceed in such a way to transmit this information to clavulanic acid (**1**). The suggested intermediacy of lactate[8] or pyruvate,[10,12] therefore, can be excluded; that is, isotopic labels which are diastereotopic in **4** and **5** become achiral in a methyl group and, consequently, lose their ability to transfer label stereospecifically to clavulanic acid.

Two further observations made it possible to establish the stereochemical course of N−C bond formation in the construction of CEA (**3**). First, it could be shown that both H_A and H_B in glycerol (**4**) are completely retained in the formation of proclavaminic acid (**6**).[13] Second, incubation of **5** stereospecifically labeled at C-4′ (**5**, H_A or H_B) with clavaminate synthase demonstrated that the oxidative cyclization/desaturation to clavaminic acid (**7**) occurred with clean stereochemical retention.[14] Knowing H_B survives the striking ring inversion to clavulanic acid (**1**), a complete stereochemical correlation could now be deduced as outlined in Scheme 1. It may be concluded that the C−O bond in **4** and **5** is replaced by the N−C bond in CEA (**3**) with overall retention of configuration. In sum, these results limit the possible precursor from primary metabolism to a C_3-carbohydrate likely lying between glycerol and PEP whose hydroxymethylene oxidation state is maintained throughout stereospecific CEA formation.

Scheme 1



Identification of the 3-carbon unit became possible with the discovery of a new biosynthetic enzyme that cyclizes CEA (**3**) to the β-lactam ring contained in proclavaminic acid (**6**, Scheme 1). This ATP/Mg^{2+}-dependent protein catalyzes a previously unknown reaction type, a β-lactam synthetase, and is encoded by the second gene in the clavulanic acid biosynthetic cluster.[15,16] The first gene of the cluster lies directly upstream and gives rise to a protein (60907 Da) showing sequence identities as high as 29% to acetolactate synthases from several sources, and to a lesser extent to pyruvate oxidases. The potential relation of a thiamine pyrophosphate-dependent enzyme such as these to any step in clavulanic acid biosynthesis was not obvious.

To examine the biosynthetic role of its encoded gene, *orf2* was cloned into pET24a (Novagen) and used to transform *E. coli* B834(DE3). As a control, this host was also transformed with the vector alone. The recombinant clones were separately inoculated into LB medium and, once growth had reached A_{600} = 0.7, they were transferred to sterile flasks and induced with IPTG. After 3 h, 1 mM [U-^{14}C]arginine (50 μCi/mmol) was added and incubation was continued for an additional 21 h at 28 °C. The cells were harvested by centrifugation and the supernatants were analyzed by HPLC after microfiltration. The appearance of radioactivity in the chromatograms was monitored by scintillation counting. Significant radioisotope was detected in samples with a retention time coincident with CEA in the sample from the recombinant bearing *orf2*, but not the control culture. This finding

* Address correspondence to this author. E-mail: Townsend@jhunix.hcf.jhu.edu.

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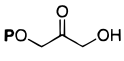
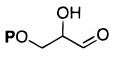
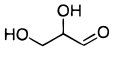
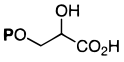
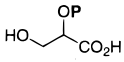
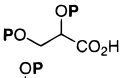
10.1021/ja9923134 CCC: $18.00
Published on Web 09/16/1999

Table 1. Screening of Potential C3-Carbohydrates with Either a Cell-Free Extract (CFE) of *E. coli* B834(DE3) Overexpressing *orf2*, or Dialyzed 30% Ammonium Sulfate Pellet Containing >95% CEA Synthase (See Text)

structure	abbrev.	conc. (mM)	% conversion CFE	% conversion 30% $(NH_4)SO_2$ pellet
	DHAP	30	9.7	29.8
	D,L-G3P	30	3.1	3.9
		60	-	6.1
	D,L-GA	30	3.8	2.4
	3-PGA	30	3.5	0.5
	2-PGA	30	4.6	1.2
	2,3-DPGA	30	3.4	0.3
	PEP	30	2.8	2.6
	PA	30	3.4	1.1

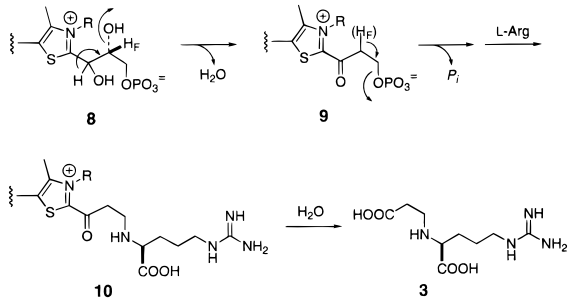
implied that Orf2 catalyzed the condensation of L-arginine with some primary metabolite available in *E. coli* to synthesize CEA (**3**), that is, the elusive C_3-unit itself.

Preliminary identification of the precursor of the C_3-unit was sought in an in vitro experiment. A cell-free extract (CFE) was prepared from frozen cell paste of the recombinant strain according to the method of Busby et al.[17] [U-^{14}C]Arginine was incubated in the presence of various potential C_3-intermediates (30 mM), TPP (1.5 mM), and the CFE in Tris buffer. The glycolytic intermediates examined were several phosphoglyceric acids (PGA, Table 1), D,L-glyceraldehyde-3-phosphate (D,L-G3P), dihydroxyacetone phosphate (DHAP), phosphoenolpyruvate (PEP), D,L-glyceraldehyde (D,L-GA), and pyruvic acid (PA). After 3 h of reaction at room temperature, the protein was removed by membrane filtration (Ultrafree 5000, Millipore) and the samples were analyzed by HPLC and scintillation counting as before. Despite our fear that extensive equilibration among the intermediates of glycolysis would cloud the outcome, the results of this experiment were clear, if unexpected. Although low levels of radioactivity appeared in CEA in every trial, the most efficient production of this first biosynthetic intermediate was observed with DHAP (Table 1).

As a first step toward purifying CEA synthase, it was discovered that a fortuitously efficient precipitation of the overproduced enzyme could be carried out with 30% ammonium sulfate. The protein pellet was resuspended and dialyzed[18] to give substantially pure CEA synthase (>95%) when examined by SDS-PAGE. Rescreening of the glycolytic intermediates with the partially purified enzyme gave reduced background counts in CEA and appeared to confirm the identity of DHAP as the precursor of the β-lactam carbons of clavulanic acid from primary metabolism (Table 1). A large-scale incubation of DHAP and L-arginine provided further evidence of this remarkable reaction yielding a single product whose chromatographic behavior and ^{1}H NMR spectrum were identical with an authentic specimen of CEA (**3**).[15]

However, reevaluation of D,L-G3P at higher concentration (60 mM) gave a greater incorporation into CEA (**3**) (Table 1).

Scheme 2



Suspecting that one or the other enantiomer of G3P might be inhibitory, D-G3P was generated and found to give a conversion to CEA comparable to that of DHAP. It now appeared, therefore, that both DHAP and D-G3P could serve as substrates for the enzyme in the synthesis of CEA.

Triosephosphate isomerase (TIM) mediates the isomerization of DHAP and G3P and is notorious for its exceptionally high catalytic activity.[19] Even a slight contamination by this enzyme could be responsible for the DHAP/D-G3P interconversion apparently carried out by CEA synthase. To examine this possibility, the substantially pure solubilized 30% ammonium sulfate pellet was loaded onto an L-arginine−agarose affinity column (Sigma) and eluted with a gradient of NaCl to give a highly purified sample of CEA synthase as judged by SDS-PAGE. The conversion of DHAP to D-G3P in a standard TIM assay[20] carried out in the presence of the affinity-purified synthase, but in the absence of L-arginine, was significantly less efficient (<0.1%) than that from the 30% ammonium sulfate pellet (ca. 20%). Importantly, while the specific activities of the time-dependent transformation of D-G3P + [^{14}C]-L-arginine to CEA (**3**) in the presence of TPP increased as the purification of the protein advanced, the conversion of DHAP, while initially high in the CFE, fell successively to background when assayed with the affinity purified enzyme. Another round of purification through the affinity step gave homogeneous protein by SDS-PAGE and essentially unchanged activities for these two substrates indicating the trace TIM activity had been removed.

While further experiments remain, D-G3P (**2**) is proposed to undergo addition by the thiamine pyrophosphate ylide to form **8** (Scheme 2). β-Elimination of hydroxide and ketonization to **9** may then be invoked, whereupon elimination of phosphate followed by the addition of L-arginine with retention of configuration at the β-carbon, as required by earlier stereochemical experiments,[13,14] is proposed to give **10**. Presumably loss of H_F in 3-phosphoglycerate (**5**) takes place from D-G3P at this stage. Finally, addition of water would lead to release of the product, N^2-(2-carboxyethyl)arginine (**3**), and regeneration of the resting state of the enzyme. Thiamine pyrophosphate is absolutely required, in keeping with the translated signature motif noted in *orf2*. This is an unusual transformation for this cofactor more commonly associated with C−C bond-breaking and bond-forming reactions as, for example, transketolases or the decarboxylation of α-ketoacids. CEA synthase mediates an internal redox reaction and a β-elimination/addition leading to N−C bond formation in the synthesis of **3**. This is a pleasingly adroit process in which the carboxyethyl of the product **3** required for β-lactam formation is generated by the capture of a glycolytic intermediate having the equivalent oxidation state.

Acknowledgment. We are grateful to the NIH (AI14937) for financial support and to Brian O. Bachmann (JHU) for early insight into the mechanism of this reaction. This paper is dedicated to Professor D. Arigoni (ETH, Zürich) for his abiding interest in this problem.

JA9923134

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