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Stereochemical Studies on Phosphopantothenoylcysteine Decarboxylase from *Escherichia coli*

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Abstract—Phosphopantothenoylcysteine decarboxylase catalyzes the decarboxylation of 4′-phosphopantothenoylcysteine (**2**) to form 4′-phosphopantetheine (**3**), an intermediate in the biosynthesis of Coenzyme A. In this study we investigated the stereochemistry of this reaction. Our results show that the decarboxylation proceeds with retention of stereochemistry, and that the pro-*R* proton at C_β of the cysteine moiety of **2** is removed during a reversible oxidation of the thiol to a thioaldehyde intermediate.

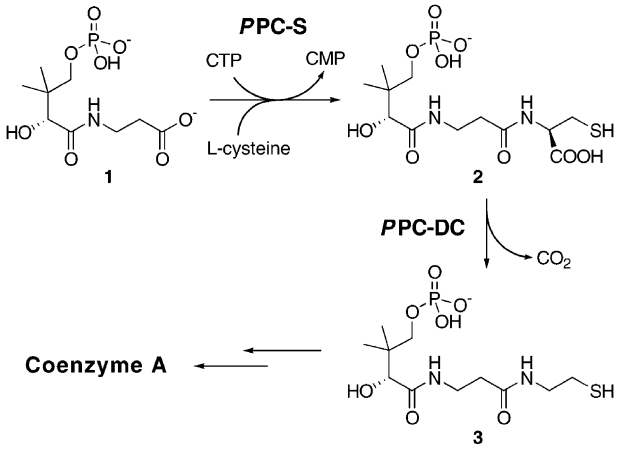
Phosphopantothenoylcysteine decarboxylase (*P*PC-DC) catalyzes the decarboxylation of the cysteine moiety of 4′-phosphopantothenoylcysteine (**2**, *P*PC) to form 4′-phosphopantetheine (**3**).[1,2] In *Escherichia coli* and most other prokaryotes (with the exception of the Enterococci and Streptococci) this activity is fused with another enzyme bearing phosphopantothenoylcysteine synthetase (*P*PC-S) activity to form a bifunctional enzyme (CoaBC),[1] while *P*PC-DC enzymes in eukaryotic systems, including *Arabidopsis thaliana*[3] and the recently identified human enzyme,[4] are monofunctional (Scheme 1). Both of these activities form part of the biosynthetic machinery that converts pantothenate (Vitamin B_5) into Coenzyme A (CoA), an essential cofactor in all biological systems.

Previously it has been shown that *P*PC-DC is a flavoenzyme, and that the decarboxylation reaction is likely to proceed via a thioaldehyde intermediate (**6**) formed by flavin-catalyzed oxidation of the thiol of the cysteine moiety of **2** (Scheme 2).[5] Our current understanding of the mechanism does not differentiate between hydride transfer as shown in Scheme 2 and a sequential single electron transfer (SET) mechanism.

In this study we describe the stereochemical course of the reaction catalyzed by the recombinant enzyme from *E. coli* at both C_α and C_β of the cysteine moiety of **2**. The stereochemistry of the partially purified rat liver enzyme has previously been reported, showing decarboxylation with retention of configuration.[6] This work provides further insight into the active site environment and the nature of the catalytic mechanism of this interesting and biosynthetically essential enzyme.

Analysis of Stereochemistry at C_α

To determine the stereochemical course of the decarboxylation reaction at C_α of the cysteine moiety of **2**, L-2-methyl-cysteine (**11**)[7] was used as an alternate



Scheme 1.

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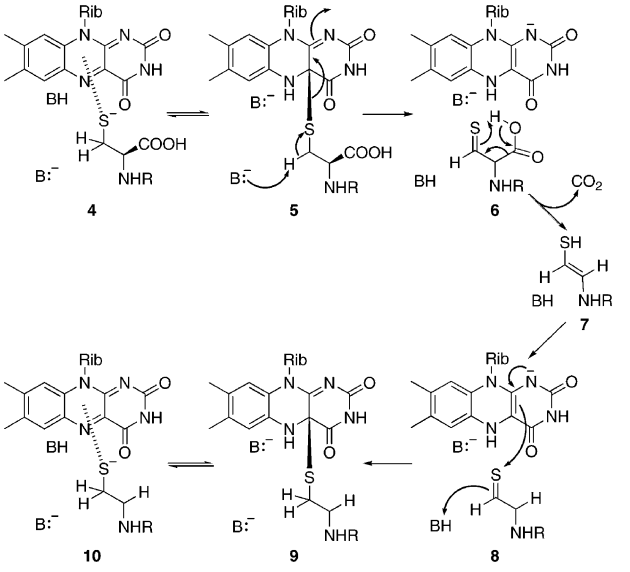
substrate in the reaction catalyzed by *P*PC-S. In this way the product **12**, in which the α-proton has been replaced with a methyl group, is formed—this compound can then serve as an alternate substrate for *P*PC-DC. Indeed, when **11** was incubated with the co-substrates CTP and 4′-phosphopantothenate (**1**) in the presence of CoaBC, the methylated 4′-phosphopantetheine analogue **13** was detected as the sole product of reaction as judged by ESI-MS analysis of the reaction mixture (Scheme 3). Compound **13** was partially purified by cation exchange chromatography to remove buffer components and excess **11**, and subsequently treated with alkaline phosphatase. The dephosphorylated product **14** was exhaustively extracted from the reaction mixture, and reacted with the fluorescent chiral tagging reagent (*R*)-(−)-4-(3-isothiocyanatopyrrolidin-1-yl)-7-(*N*,*N*-dimethylamino-sulfonyl)-2,1,3-benzoxadiazole (*R*-(−)-DBD-PyNCS, **15**) (Scheme 4). This reagent has previously been used for the resolution of thiol enantiomers as diastereomeric derivatives by reverse-phase HPLC.[8] The stereochemical identity of the resulting diastereomer **16** can then be determined by co-injection with (2′*R*)-**16** and (2′*S*)-**16**, prepared by derivatization of (2′*R*)-**14** and (2′*S*)-**14** independently synthesized as shown in Scheme 5. The resulting chromatograms are shown in Figure 1, and indicate that the labeled product **16** produced by the enzymatic decarboxylation of **12** co-elutes with (2′*S*)-**16**, but is separated from (2′*R*)-**16**. These results show that the enzyme produced (2′*S*)-**13**, and thus we conclude that the decarboxylation reaction proceeds with retention of stereochemistry.

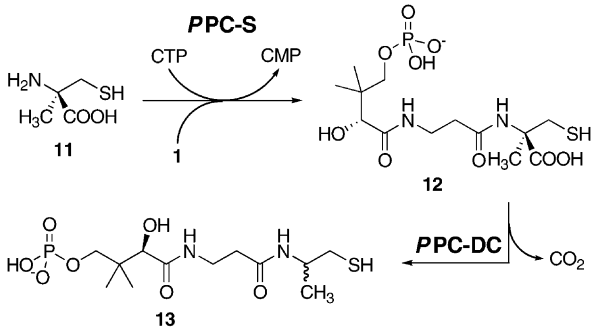
Analysis of Stereochemistry at C_β

Our initial attempts to determine the stereochemistry of the reaction at C_β of the cysteine moiety involved making use of the two diastereomers of L-3-methyl-cysteine, synthesized by replacing the alcohol of L-threonine and L-*allo*-threonine with a thiol in a strategy similar to that employed in the synthesis of **14** (Scheme 5).[10] However, this approach had to be abandoned since CoaBC did not accept L-3-methyl-cysteine as an alternate substrate. Thus a different strategy had to be sought in which the stereoselectivity could be determined using alternate substrates which are sterically less intrusive.

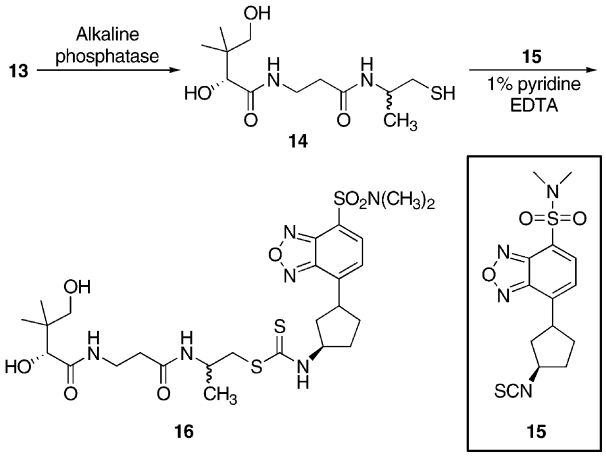
Since we had previously successfully measured the primary deuterium isotope effect at C_β of the cysteine



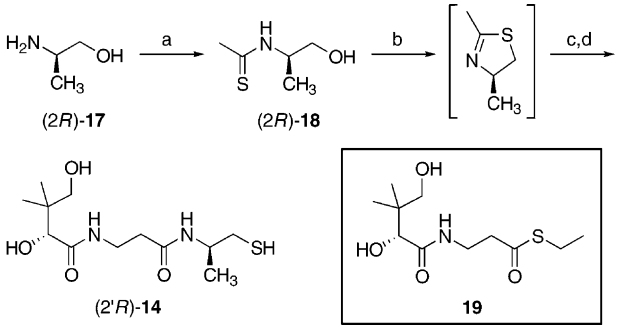
Scheme 2.



Scheme 3.

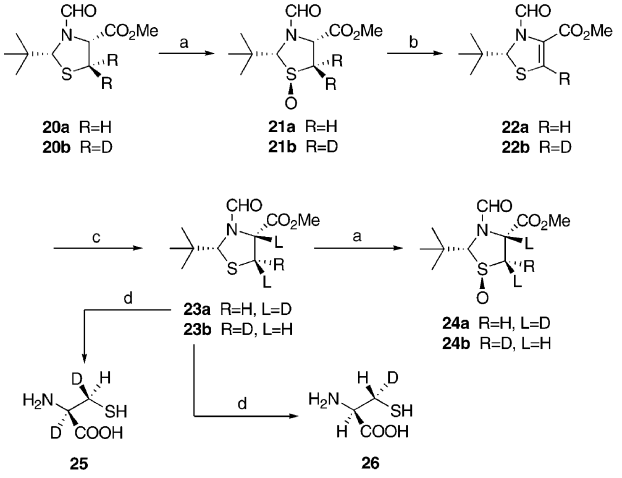


Scheme 4.



Scheme 5. Synthesis of authentic (2′*R*)-**14** from the appropriate propanolamine. (2′*S*)-**14** was synthesized in a identical fashion using the (*S*)-enantiomer. Reagents and conditions: (a) ethyldithioacetate, MeOH, reflux, 16 h, 90%; (b) (diethylamino)sulfur trifluoride, CH_2Cl_2, −78 °C; (c) 3 M HCl, 100 °C; (d) **19**,[9] DIPEA, MeOH, reflux, 3 h, 31% overall.

moiety of **2** ($^{D}(V/K) = 1.81 \pm 0.04$),[5] making use of L-3,3-[^{2}H]$_2$-cysteine, we set out to repeat the same experiment using L-cysteines stereospecifically deuterated at C_β. Our proposed mechanism (Scheme 2) predicts that only one of the hydrogen atoms at this center is removed during the course of the reaction - consequently we would expect to observe a larger primary deuterium isotope effect for one hydrogen atom at C_β than for the other. However, to perform this experiment the synthesis of the two diastereomers of L-3-[^{2}H]-cysteine is required. Although various stereospecific syntheses have previously been published,[11] we decided to use variations on this chemistry as outlined in Scheme 6, starting from the thiazolines **20**.[7,12] This scheme results in one diastereomer also being deuterated at C_α— however, since we have shown that the C_α–H bond is not cleaved during the course of the reaction, it is unlikely that this substitution would perturb the relative magnitude of the isotope effects at C_β. Finally, the stereochemical purity of the deuterated cysteines produced in this manner was assessed by NMR analysis of the immediate precursors of these compounds, the thiazolines **23**, after conversion to their *S*-oxides **24**. This allows the resolution of the relevant signals as shown in Figure 2.



Scheme 6. Synthesis of (2*R*,3*R*)-2,3-[^{2}H]$_2$-cysteine and (2*R*,3*S*)-3-[^{2}H]-cysteine. Reagents and conditions: (a) H_2O_2, AcOH, 89%; (b) TBDMSOTf, Et_3N, CH_2Cl_2, 6 h, 34%; (c) H_2 or D_2, 10% Pd/C, 65-77%; (d) 6 M HCl, quantitative.

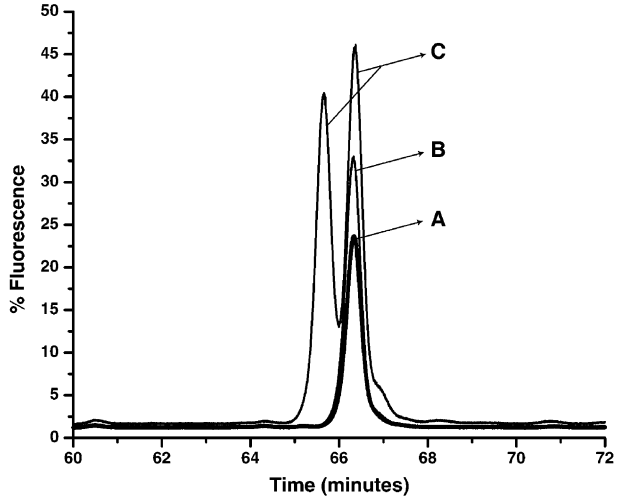


Figure 1. Determining the stereochemical course of the decarboxylation reaction catalyzed by *P*PC-DC. **A**: Chromatogram of the labeled enzyme product. **B**: The labeled enzyme product co-injected with (2′*S*)-**16**. **C**: The labeled enzyme product co-injected with (2′*R*)-**16**. Unreacted **15** eluted at 99 min, and an unidentified component at 56 min.

We measured the $^{D}(V/K)$ isotope effects at C_β for the decarboxylation reaction by treating an equimolar mixture of L-cysteine and either L-3,3-[^{2}H]$_2$-cysteine, (2*R*,3*R*)-2,3-[^{2}H]$_2$-cysteine (**25**) or (2*R*,3*S*)-3-[^{2}H]-cysteine (**26**) with CoaBC in the presence of CTP and **1** and measuring the relative deuterium content of the product **3** by ESI-MS analysis both after partial (< 5%) and complete conversion. The isotope effects were calculated by dividing the ratio of deuterated and non-deuterated products at partial conversion by the ratio of the same at complete conversion, as previously described.[5] The data are summarized in Table 1.

While the results demonstrated a small isotope effect at the pro-*S* position on C_β, the effect at the pro-*R* position was relatively large and comparable to the effect when both hydrogen atoms at C_β were substituted with deuterium. We thus conclude that it is only the pro-*R* proton that is removed during the course of catalysis.

Mechanistic Implications and Predicted Active Site Architecture

Our stereochemical analysis suggests the active site geometry shown in Scheme 7. In this model, retention of

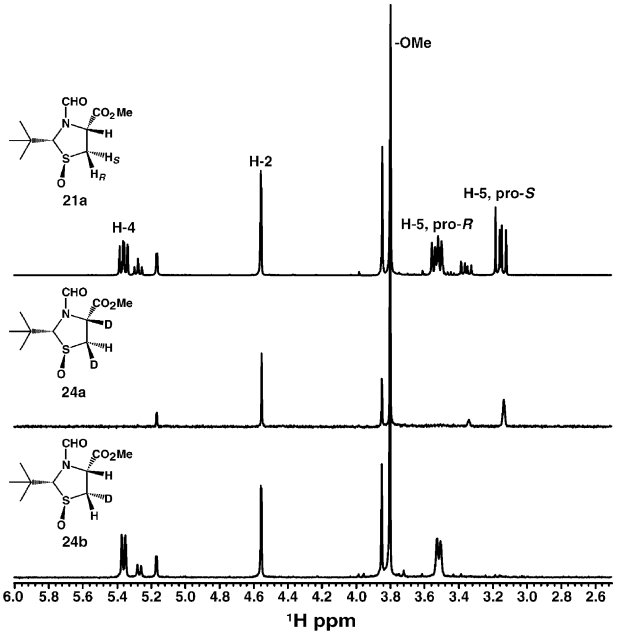
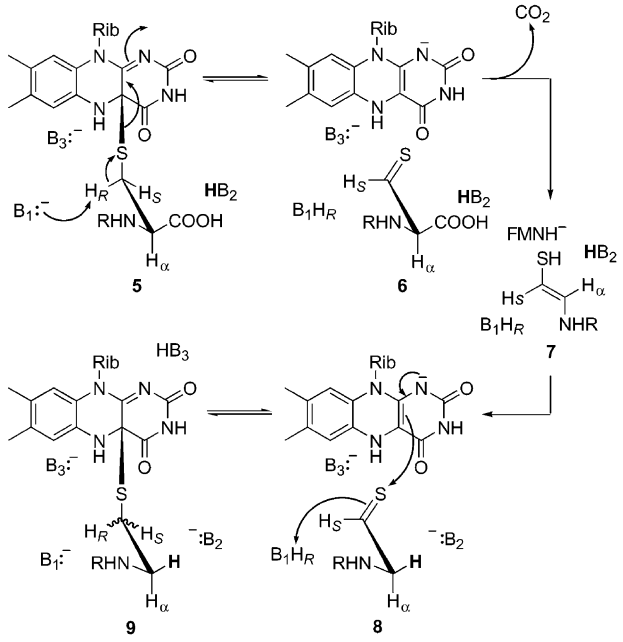


Figure 2. Confirmation of the absolute configuration of the deuterated cysteines **25** and **26**. The thiazolidine precursors **23** were converted to the *S*-oxides **24**, and their ^{1}H NMR spectra compared to the unlabeled *S*-oxide **21a**. Each molecule with the relevant region on its NMR spectrum is shown. The signals arising from the hydrogen atoms at C-5 of **21a** was distinguished by NOESY analysis. Note the presence of minor peaks due to the existence of a second conformer of each molecule in solution.

Table 1. Primary deuterium isotope effects on the decarboxylation of **2** as catalyzed by CoaBC[a]

Cysteine used	$^{D}(V/K)$
L-3,3-[^{2}H]$_2$-Cysteine	1.89±0.03
(2*R*,3*R*)-2,3-[^{2}H]$_2$-Cysteine (**25**)	1.85±0.03
(2*R*,3*S*)-3-[^{2}H]-Cysteine (**26**)	1.15±0.04

[a]The isotope effects was determined in direct competition reactions using equimolar mixtures of non-deuterated L-cysteine and the relevant cysteine stereospecifically deuterated at C_β. See the text for details.



Scheme 7.

stereochemistry at C_α results when an active site acid (B_2H), positioned on the same side of the substrate as the departing carboxylate, serves as the proton donor of the decarboxylated intermediate, while an active site base (B_1) removes the pro-*R* hydrogen atom at C_β during the oxidative formation of **6** as shown. Alternatively, the pro-*R* hydrogen atom can be transferred to N-5 of flavin. Since the hydrogen atom removed from C_β is subsequently returned to the same center upon reduction of **8** (i.e., no solvent exchange is observed during the course of the reaction),[5] it is not possible for a single residue to carry out the functions of both B_1 and B_2H. The stereochemical course of the reduction at C_β was not determined.

While the structure of the *P*PC-DC from *A. thaliana* has been solved, no ligand is bound at the active site, making the reliable identification of the key catalytic residues difficult.[13] Although various mutational studies of residues in the proposed active site of this enzyme have been performed,[3,14] detailed analysis of these studies, also informed by the results of our current stereochemical investigation of the reaction, will have to await the solution of a structure of a *P*PC-DC enzyme co-crystallized with substrate or relevant analogues.

Acknowledgements

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9. Sodium pantothenate (2.0 g, 8.3 mmol) was exchanged to the free acid over Amberlite IR-120 (H^+) and lyophilized. The resulting residue was dissolved in DMF (10 mL), and diphenylphosphoryl azide (3.72 mL, 16.6 mmol) and ethanethiol (0.75 mL, 10.0 mmol) were added. After cooling to 0 °C triethylamine (2.3 mL) was added, the solution was stirred for 10 min at 0 °C, and then at room temperature for 2 h. Ethyl acetate was added (100 mL), and the solution was washed with 1 M HCl (2×20 mL), 1 M $NaHCO_3$ (2×20 mL) and saturated NaCl (1×20 mL). The solution was dried (Na_2SO_4) and the solvent evaporated in vacuo. The product was purified by flash chromatography on silica gel (ethyl acetate/hexane 3:1–4:1) to give **19** as a colorless oil (1.0 g, 46%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.89 (s, 3H), 0.98 (s, 3H), 1.23 (t, 3H), 2.79 (t, 2H), 2.78 (q, 2H), 3.49 (s, 2H), 3.54–3.61 (m, 2H), 4.02 (s, 1H).
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