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# Hydroxylation by Flavin Enzymes: Evidence for NIH-Shift Mechanism

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## Introduction

2-Aminobenzoate is an intermediate in many catabolic pathways. More specifically, biodegradation of tryptophan, indole acetic acid, and indole alkaloids yields 2-aminobenzoate as a common intermediate. Under aerobic conditions 2-aminobenzoate is further degraded via gentisate or catechol [1].
Rather recently, a novel pathway of 2-aminobenzoate metabolism has been elucidated. It has been shown in the eubacterium *Azoarcus evansii* that 2-aminobenzoate and coenzyme A are converted into 2-aminobenzoyl CoA by the catalytic action of a specific ligase [2]. 2-Aminobenzoyl CoA can then serve as substrate of a monoxygenase/reductase [3-6]. More specifically, the flavoprotein 2-aminobenzoyl CoA monooxygenase/reductase (ACMR) catalyzes the dearomatization of 2-aminobenzoyl CoA in a NADH- and $O_2$-dependent reaction by a hitherto unknown mechanism. The enzyme is a dimer comprising two 85 kDa polypeptide chains and contains one FAD per subunit. Based on earlier studies, ACMR contains two domains (a monooxygenase and a reductase site) each containing one FAD molecule [3-5].
In this study, the structure of the enzyme product and the mechanism of its formation are analyzed by NMR spectroscopy.

## Methods

*Organism and enzyme purification. Azoarcus evansii* (previously designated *Pseudomonas* sp. KB740) was grown and ACMR was purified as described earlier [4].

*Enzyme assay.* A mixture containing 500 µmol $KP_i$ (pH 7.8), 100 µmol NADH, 10 µmol 2-aminobenzoyl CoA, and 5.6 U ACMR in a total volume of 100 ml was incubated aerobically at 37 °C for 20 min. The enzyme was inactivated by the addition of 10 mmol methylmethanethiosulfonate and was then applied to a Waters Sep Pak $C_{18}$ column which was washed with 40 ml of 5 mM $KP_i$ (pH 7.8) and was subsequently developed with 50 % methanol in 5 mM $KP_i$ (pH 7.8). The effluent was lyophilized. Further purification was achieved by preparative HPLC using a column of $RP_{18}$ (250 x 20 mm) which was eluted with acetonitril/methanol/4.5 mM KPi (pH 7.8) (86/9.5/4.5; v/v/v). The retention volume of enzyme product was 125 ml. Fractions were lyophilized.
*NMR spectroscopy.* Enzyme product was dissolved in 19 mM $KP_i$ (pH 7.6) containing 10 % $D_2O$. $^1H$, $^2H$, and $^{13}C$ NMR spectra were recorded at 7 °C using a Bruker DRX 500 spectrometer.

## Results

ACMR was incubated with 2-aminobenzoyl CoA in the presence of $O_2$ and NADH. The product of the conversion was isolated by HPLC (yield, 2-3 mg) and analyzed by NMR spectroscopy.
*Experiment with [ring-$^{13}C_6$]2-aminobenzoyl CoA.* In an attempt to increase the sensitivity and the selectivity of NMR experiments we used [ring-$^{13}C_6$]2-aminobenzoyl CoA as substrate. The $^{13}C$ NMR spectrum of the enzyme product (Fig. 1) was dominated by six double-doublets or pseudo-triplets ($^{13}C^{13}C$ coupling constants, 30 - 70 Hz) indicating simultaneous coupling to two directly bonded $^{13}C$ atoms for each $^{13}C$ enriched atom. Thus, the connectivity of the $^{13}C$ labeled ring carbon atoms of 2-aminobenzoyl CoA is retained in the enzyme product. Two-dimensional INADEQUATE, HMBC, HMQC, and TOCSY experiments revealed the connectivities between carbon and hydrogen atoms (Table). The observed spectroscopic signature established the structure of the enzyme product as 2-amino-5-oxocyclohex-1-ene-1-carbonyl CoA (AOC, Fig. 2) and confirmed earlier proposals [4-5].

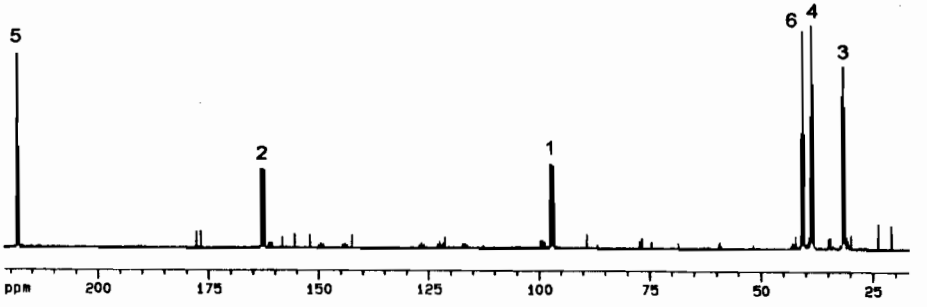


Fig. 1: $^{13}C$ NMR spectrum of [ring-$^{13}C_6$]2-amino-5-oxocyclohex-1-ene-1-carbonyl CoA.

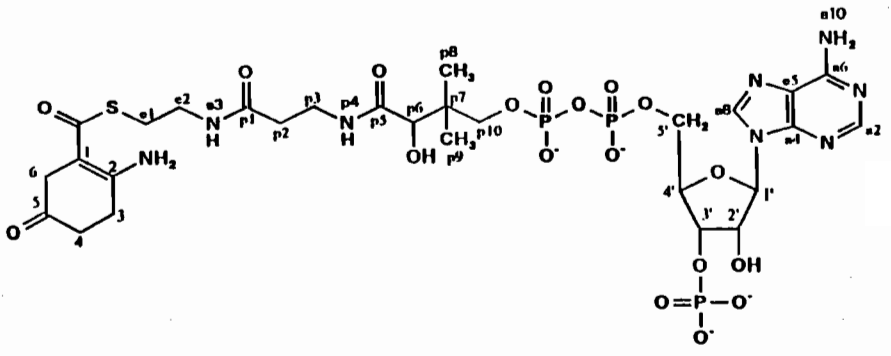

Fig. 2: 2-Amino-5-oxocyclohex-1-ene-1-carbonyl CoA (AOC), product obtained from reaction with ACMR.

The novel compound was stable for several days in phosphate buffer at neutral pH and at 7 °C. Spontaneous exchange of hydrogen atoms at C-4 and C-6 with solvent $^2H_2O$ was observed. The half-lives of exchange were 300 h and 25 h at 7 °C, and 23 h and 2h at 37 °C for H-4 resp. H-6.

Table: NMR Data of the Carbocyclic Moiety of [ring-$^{13}C_6$]2-Amino-5-oxocyclohex-1-ene-1-carboxyl CoA

| | $^1H$ ppm | $^{13}C$ ppm | $J_{CC}$ Hz | $J_{HH}$ Hz | TOCSY | HMBC |
|---|---|---|---|---|---|---|
| COSCoA | | 190.42 | | | | e1,6 |
| 1 | | 96.94 | 68.6(2),46.9(6),2.9 | | | 6,3 |
| 2 | | 162.59 | 68.4(1),40.6(3),1.8 | | | 6,3,4 |
| 3 | 2.55 (t) | 31.46 | 40.6(2),31.6(4),2.0 | 6.8 | 4 | 4 |
| 4 | 2.42 (t) | 38.61 | 39.6(5),31.7(3),10.0(6),2.9 | 6.8 | 3 | 3,6(w) |
| 5 | | 218.14 | 39.5(4),37.8(6),3.5 | | | 6,3,4 |
| 6 | 3.01 (s) | 40.65 | 46.9(1),37.7(5),10.3(4),1.8 | | | 4(w) |

*Experiment with [5-$^2$H]2-aminobenzoyl CoA.* The $^2H$ decoupled $^1H$ NMR spectrum of AOC obtained from [5-$^2$H]2-aminobenzoyl CoA showed a singlet signal for H-6 with reduced intensity as compared to the spectrum of an unlabeled sample (Fig. 3). On the other hand, an up-field shifted satellite signal of H-6 was observed. The amount of the shift (22 ppb) was indicative for a β-effect of a $^2H$ atom on the chemical shift of the adjacent H-6. In line with this, we observed a $^2H$ NMR signal at 3 ppm confirming the presence of a $^2H$ atom at C-6. Obviously, the AOC sample from [5-$^2$H]2-aminobenzoyl CoA was a mixture of unlabeled AOC and [6-$^2$H]AOC.

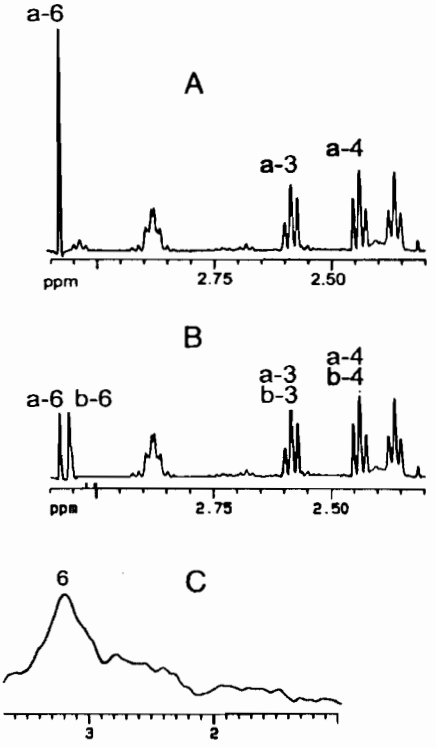


Fig. 3: A, part of a $^{1}H$ NMR spectrum of unlabeled AOC; B, part of a $^{1}H$ NMR spectrum of AOC obtained from [5-$^{2}H$]2-aminobenzoyl CoA; C, part of a $^{2}H$ NMR spectrum of AOC obtained from [5-$^{2}H$]2-aminobenzoyl CoA. Isotopomers (a,b) are assigned according to Fig. 4

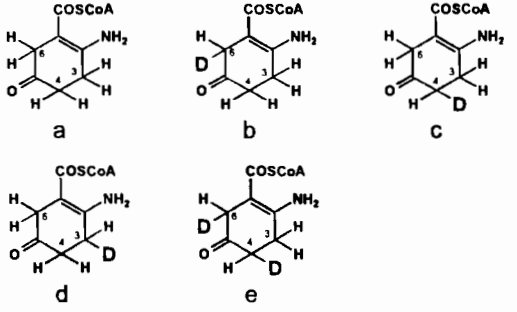


Fig. 4: $^{2}H$ labeled isotopomers (b-e) of AOC observed in this study

*Experiment with [4R-$^2$H]NADH.* The $^2$H decoupled $^1$H NMR spectrum of AOC displayed a doublet at 2.42 ppm (H-4) indicating coupling to only one hydrogen atom at C-3 (Fig. 5). Accordingly, the intensity of the triplet signal at 2.55 ppm (H-3) was reduced and a $^2$H NMR signal at 2.5 ppm was observed. These data reflected the formation of [3-$^2$H]AOC (isotopomer d, Fig. 4) by ACMR reaction in the presence of [4*R*-$^2$H]NADH. It should be noted that the reaction with [4*S*-$^2$H]NADH did not afford deuterated product.

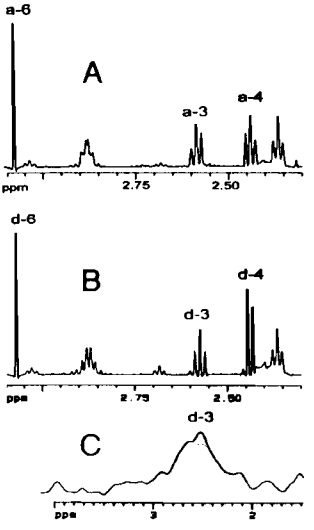


Fig. 5: A, part of a $^1$H NMR spectrum of unlabeled AOC; B, part of a $^1$H NMR spectrum of AOC obtained from [4*R*-$^2$H]NADH; C, part of a $^2$H NMR spectrum of AOC obtained from [4*R*-$^2$H]NADH. Isotopomers are assigned according to Fig. 4

*Experiment in $^2H_2O$.* AOC isolated from incubation in buffer containing $^2H_2O$ gave a doublet $^1$H NMR signal at 2.55 ppm (H-3) indicating formation of [4-$^2$H]AOC (isotopomer c, Fig. 4). In line with this, a $^2$H NMR signal was observed at 2.4 ppm. Additionally, an upfield-shifted $^1$H satellite signal was detected for H-6 and a $^2$H NMR signal was found at 3.0 ppm suggesting partial deuteration at C-6 (formation of isotopomer e). The presence of isotopomer e is explained by spontaneous hydrogen exchange at C-6 during incubation in deuterated buffer (see above).

## Discussion

The product of ACMR conversion was unequivocally assigned as 5-oxo-2-aminocyclohex-1-ene-1-carbonyl CoA (AOC) by multidimensional and multinuclear NMR analysis. Incubation of ACMR in buffer containing deuterated substrates afforded specifically labeled AOC samples. More specifically, [5-$^2$H]2-aminobenzoyl CoA was converted into [6-$^2$H]AOC (Fig. 6). This result was unexpected and compatible with a hydride shift from the 5 position of the substrate to the 6 position of the product (see below). Label from [4*R*-$^2$H]NADH was transferred to the 3 position of the cyclic enamine, whereas label from solvent $^2H_2O$ was incorporated into the 4 and the 6 position of AOC (Fig. 6).

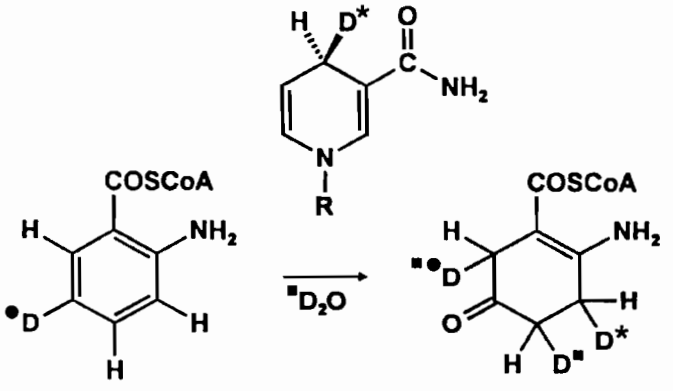


Fig. 6: Labeling pattern of AOC isolated from 2-aminobenzoyl CoA converted by ACMR

In conjunction with earlier studies [3-6] and on the basis of the observed $^2$H labeling signature (Fig. 6), a mechanism of ACMR reaction can be proposed.
First, 2-aminobenzoyl CoA is monooxygenated by the monooxygenase domain of ACMR. It is assumed that a 4a-hydroperoxide flavin is formed from $FADH_2$ in a $O_2$-dependent reaction. Nucleophilic attack of 2-aminobenzoyl CoA on the distal hydroperoxide oxygen atom with concomitant expulsion of 4a-hydroxy flavin yields a hydroxylated diene intermediate (Fig. 7A). The reaction is directed by the amino moiety of 2-aminobenzoyl CoA located para to C-5 and thus activating C-5 for nucleophilic attack.

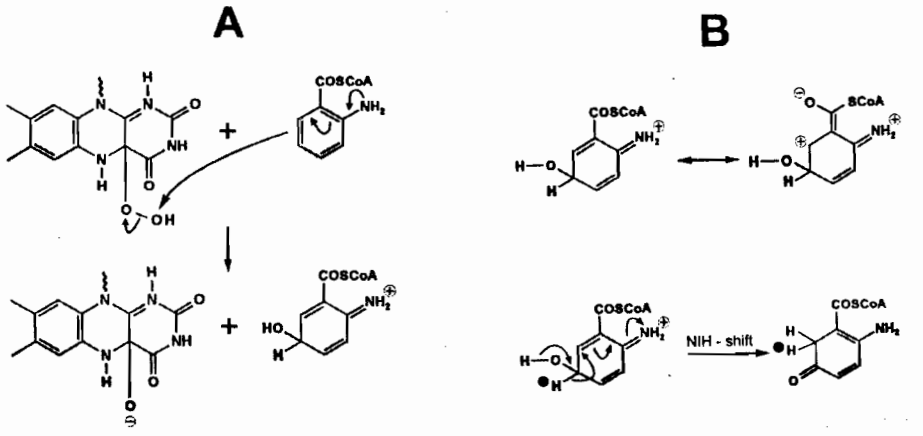


Fig. 7: Reactions catalyzed by the monooxygenase domain of ACMR.

Subsequently, a hydride is shifted from C-5 to C-6 of the diene intermediate yielding 5-oxo-2-aminocyclohex-1,3-diene-1-carbonyl CoA (Fig. 7B, below). This shift can proceed in a concerted process, as formulated originally for the so-called NIH shift. Notably, hydride migration occurs toward C-6 and not toward C-4. This regiochemical course can be explained by the additional activation of C-6 through the electron withdrawing effect of the adjacent CoA residue (Fig. 7B, top).
Under limiting NADH concentrations, 5-oxo-2-aminocyclohex-1,3-diene-1-carbonyl CoA is rearomatized to 2-amino-5-hydroxybenzoyl CoA [6]. On the other hand, in the presence of NADH the reactive ketodiene intermediate is trapped at the reductase domain of ACMR. More specifically, the *pro-R* hydrogen atom of NADH is transferred to the flavin cofactor which serves then as reductant of 5-oxo-2-aminocyclohex-1,3-diene-1-carbonyl CoA. Most probably, the reduction proceeds by a Michael-like addition of a hydride to C-3 of the ketodiene intermediate (Fig. 8A). It should be noted that no loss of label was observed in the experiment with [4*R*-$^2$H]NADH in spite of the fact that the label is assumed to be transferred to N-5 of FAD where it could theoretically exchange with the solvent. On the other hand, limited exchange of the N-5 $FADH_2$ hydrogen atom with solvent was documented for many flavoenzymes and was explained by reduced accessibility of solvent to the cofactor in the active center [7].

Incorporation of label from solvent $^2H_2O$ into C-4 of AOC neccessarily occurs when the enolate of AOC is protonated and tautomerized into the keto form of AOC (Fig. 8B). In summary, the transformation of 2-aminobenzoyl CoA catalyzed by ACMR provides evidence for a novel NIH-shift mechanism in a flavin-mediated monoxygenation.

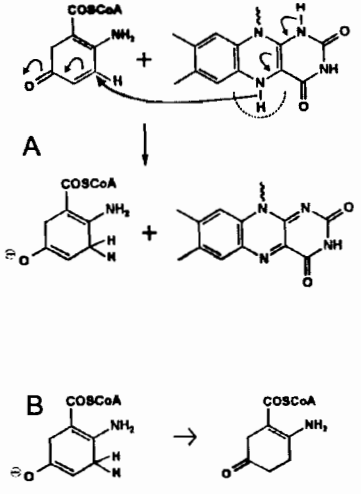


Fig. 8: Reactions catalyzed by the reductase domain of ACMR.

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