First publ. in: European Journal of Biochemistry 185 (1989), 3, pp. 637-643

2-Aminobenzoyl-CoA monooxygenase/reductase, a novel type of flavoenzyme

Studies on the stoichiometry and the course of the reaction

Reiner BUDER [1], Klaus ZIEGLER [1], Georg FUCHS [1], Bernd LANGKAU [2] and Sandro GHISLA [2]

[1] Abteilung Angewandte Mikrobiologie, Universität Ulm, FRG
[2] Fakultät Biologie, Universität Konstanz, FRG

The reaction catalyzed by 2-aminobenzoyl-coenzyme-A monooxygenase/reductase from a denitrifying *Pseudomonas sp.* has been investigated. 2-Aminobenzoyl-CoA and 2-amino[*carboxy*-^{14}C]benzoyl-CoA were synthesized enzymatically using 2-aminobenzoyl-CoA synthetase from the same organism. The product was purified by chromatography and characterized by ultraviolet/visible and ^{1}H-NMR spectroscopy. The conversion of 2-aminobenzoyl-CoA catalyzed by the monooxygenase/reductase requires NADH and oxygen, and yields at least two different products depending on the relative concentration of NADH. At [NADH] < K_m (40 μM), i.e. [NADH]/[2-aminobenzoyl-CoA] ≈ 0.02 − 0.05, the main product is probably a hydroxylated derivative of 2-aminobenzoyl-CoA, which is characterized by an absorbance maximum around 375 nm. When [NADH]/[2-aminobenzoyl-CoA] ≈ 2 − 5, the predominant product is a non-aromatic coenzyme A thioester (λ_{max} ≈ 320 nm). The stoichiometry in this case is 2.1 − 2.4 mol NADH oxidized (mol oxygen consumed)$^{-1}$ (mol 2-aminobenzoyl-CoA metabolized)$^{-1}$. The product is extremely unstable in the acidic pH range and undergoes decarboxylation in a few minutes at pH < 5. Some degree of stabilisation is obtained upon reduction with sodium borohydride, probably resulting in a further reduced non-aromatic coenzyme-A thioester.

The purification and some basic properties of a novel flavoenzym from a denitrifying *Pseudomonas* species have been described in the preceding paper [1]. This enzyme is induced during aerobic growth with 2-aminobenzoate as sole carbon and electron source suggesting that it catalyzes an initial reaction of a new bacterial pathway of aromatic compound degradation. In this organism 2-aminobenzoyl-CoA is metabolized rather than 2-aminobenzoate [2, 3]. The requirement of the three substrates 2-aminobenzoyl-CoA, NADH and oxygen suggests that the novel enzyme belongs to the monooxygenase class. For this category of flavin dependent enzymes the stoichiometry should be 1 mol NADH oxidized/mol oxygen consumed. However, NADH was required in a higher stoichiometry suggesting either nonproductive side reactions, or a hitherto unknown hydrogenation process in addition to the monooxygenase reaction itself [4].

In this work we describe the synthesis and characterization of the substrate of the enzyme, 2-aminobenzoyl-CoA, and of the corresponding ^{14}C(carboxylate)-labelled species, which were required for the study of the reaction stoichiometry. The course of the reaction and the relative amounts of products formed were studied under varying conditions, such as the concentrations of the three substrates and some of the spectral (ultraviolet/visible absorption, and ^{1}H-NMR) properties of the product mixtures are described.

Correspondence to Georg Fuchs, Abteilung Angewandte Mikrobiologie, Universität Ulm, Postfach 4066, D-7900 Ulm, Federal Republic of Germany

Enzymes. 2-Aminobenzoyl-CoA monooxygenase/reductase (EC 1.14.99.–).

MATERIALS AND METHODS

Materials

Biochemicals and enzymes were from Boehringer (Mannheim, FRG); CoA grade I was used, which was found to be anomerically pure in contrast to lower grade products, which can contain up to 30% of the α-anomer as analyzed by NMR spectroscopy. [^{14}C]KCN was from Amersham Buchler (Braunschweig, FRG); XAD 4 adsorption resin analytical grade, 50 − 100 μm, from Serva (Heidelberg, FRG); Sepralyte-cyclohexyl from ICT (Frankfurt, FRG); Sephacryl S-200 from Pharmacia Fine Chemicals (Freiburg, FRG). All chemicals used were of the highest available purity. Rotiscint 2200 was from Roth KG (Karlsruhe, FRG). D_2O was from Riedel-de Haën (Seelze, FRG).

Growth of bacteria

The denitrifying *Pseudomonas* strain KB 740$^-$ was grown on 2-aminobenzoate as sole carbon source, either under aerobic conditions at 30 °C or anaerobically with nitrate as electron acceptor at 37 °C, as has been described in a previous report [5].

Instrumentation

^{1}H-NMR spectra were recorded with a JEOL GX 400 (400 MHz) spectrometer at 25 °C. The HDO signals have been suppressed by irradiation techniques. For calibration the ^{1}H signal, *g* (4.00 ppm), was used as internal standard since a literature survey indicates that its chemical shift is largely independent of concentration and temperature in D_2O [6].

The assignment of signals of the coenzyme-A moiety is as described elsewhere [7]. Ultraviolet spectroscopy was done with a Kontron UVIKON 810 instrument.

*Synthesis of 2-amino[*carboxy-^{14}C*] benzoate*

2-Amino[*carboxy*-^{14}C]benzoate, with a specific radioactivity of 7.6 MBq/mmol was synthesized from [^{14}C]KCN and *o*-nitroaniline [8] and purified by extraction with diethyl ether and sublimation at 120°C. The ultraviolet and infrared spectra and the melting point (145°C) were identical to those of authentic 2-aminobenzoate.

Enzyme purification and assay

2-Aminobenzoyl-CoA oxidoreductase was purified, and the activity measured as described by Buder et al. 1989 [1].

Determination of NH_3

Ammonia was determined enzymatically [9].

*2-Aminobenzoyl-CoA and 2-amino[*carboxy-^{14}C*]benzoyl coenzyme A*

2-Aminobenzoyl-CoA ligase from the same *Pseudomonas*, grown aerobically or anaerobically with nitrate, was partially purified by ammonium sulfate precipitation and gel filtration as described in detail in [3], and used for the synthesis of substrates starting with unlabelled or with ^{14}C(carboxylate)-labelled 2-aminobenzoate. For the latter, the incubation mixture contained 60 mg $MgCl_2 \cdot 6\,H_2O$; 4.7 mg dithioerythritol, 42 mg coenzyme A (grade I), 91 mg ATP and 5 mg 2-amino-[*carboxy*-^{14}C]benzoate (7.6 MBq/mmol) in 150 ml 0.1 M Tris/HCl, pH 7.8. The reaction was started by adding 0.3 ml of the enzyme (1.5 mg protein; 0.75 µmol 2-aminobenzoyl-CoA formed/min at 37°C) and was followed spectrophotometrically at 365 nm. For the preparation of unlabelled product the incubation was scaled up 10-fold. After completion of the reaction, the pH was adjusted to 4–5 with 0.1 M acetic acid, the solution was centrifuged and 2-aminobenzoyl-CoA was purified from the clear supernatant by XAD 4 adsorption chromatography and preparative HPLC (see below). The lyophilized sample was a faint yellow powder. The pK_a value of the 2-amino group was determined spectrophotometrically by stepwise addition of 6 M HCl to a 100-µM solution of 2-aminobenzoyl-CoA buffered with 100 mM sodium citrate.

Preparative incubation of 2-aminobenzoyl-CoA with 2-aminobenzoyl-CoA monooxygenase/reductase and purification of product(s)

The incubation was carried out at 37°C and contained 0.2 mM 2-aminobenzoyl-CoA and 0.7 mM NADH in 500 ml 50 mM phosphate buffer, pH 7.8. The reaction was started by addition of 12 mg purified enzyme and was followed spectrophotometrically at 365 nm in a flow-through cell. After completion, the thioesters were adsorbed onto a Sepralyte-cyclohexyl column (4.4 cm × 2.5 cm), the column was washed with 200 ml phosphate buffer, pH 6.7, and the products were eluted with distilled water (detection at 254 nm). Pooled fractions were lyophilized and frozen in liquid nitrogen. A final purification can be obtained by preparative HPLC (see below). Samples were treated with sodium borohydride by addition of approximately 0.5 mg solid $NaBH_4$/ml solution of the products.

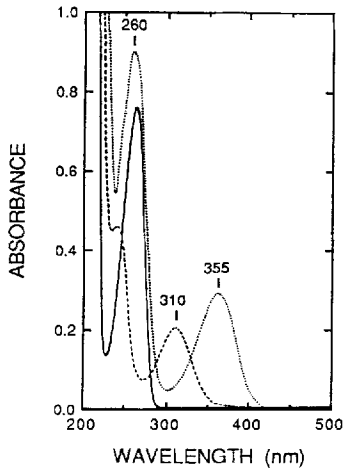


Fig. 1. *Ultraviolet absorption spectra of coenzyme A ( ), 2-aminobenzoate (– – –) and 2-aminobenzoyl-CoA (·····) each 50 µM measured in potassium phosphate buffer pH 7.0*

Reaction course under limiting concentrations of NADH or oxygen

NADH limiting conditions were set up in incubations carried out directly in optical cuvettes, in which catalytic amounts of NADH were regenerated using formate dehydrogenase (Boehringer) and formate and which contained 0.1 mM 2-aminobenzoyl-CoA, 0.5–1.0 mM formate, 0.13 U formate dehydrogenase, 2–5 µM pyrazole, 0.04–0.08 U monooxygenase/reductase in 1 ml 50–100 mM sodium phosphate buffer, pH 7.8. [NADH] was 2 µM, 5 µM, or 25 µM. The course of the reaction was followed by recording ultraviolet/visible spectra at time intervals. The stoichiometry of the reaction under a limiting supply of oxygen was determined from incubations at 21°C in which the other substrates were in excess. Thus, 0.6 ml of an incubation in a stoppered microcuvette was equilibrated at 21°C with air, and with 10% or 20% air/nitrogen mixtures; the concentration of oxygen was derived as described previously [3]. The reaction was started by addition of enzyme and was followed spectrophotometrically at 365 nm. After completion the enzyme was inactivated by addition of a solution of $AgNO_3$ to give a final concentration of 10 µM. The amount of NADH and 2-aminobenzoyl-CoA consumed was determined by HPLC using authentic samples as standards.

Chromatography

Analytical HPLC was done using an RP–18 LiChrosorb 25 cm × 0.4 cm column [2]. For large scale preparations a 25 cm × 2.0 cm RP-18 LiChrosorb column was employed, using 81.5% (by vol.) 50 mM phosphate buffer, pH 6.7, 6.5% (by vol.) acetonitrile and 12% (by vol.) methanol as mobile phase. Separation of the thioesters was complete within 15 min at a flow rate of 9.9 ml/min. TLC of the product of maleimide reduction was carried out on 0.25-mm thick aluminum oxide layers (Merck, Darmstadt, FRG) using chloroform/diethyl ether (7:3) or dichloromethane as mobile phase and subsequent detection in an iodine chamber.

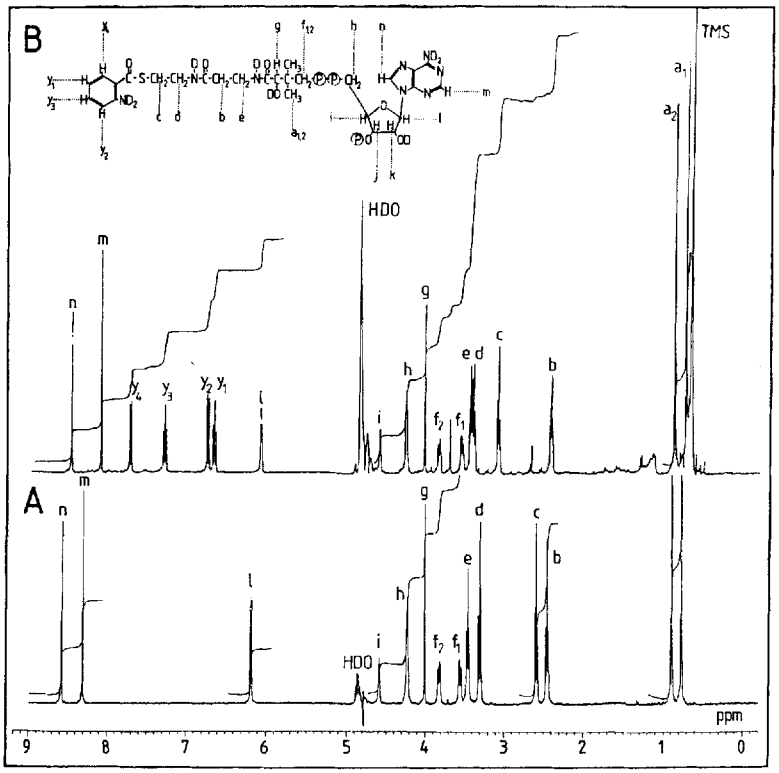


Fig. 2. *^{1}H-NMR spectra of coenzyme A (0.5 ml, 8 mM; A) and of 2-aminobenzoyl-CoA (0.5 ml, 15 mM; B).* Both compounds were lyophilized and redissolved in D_2O (pD = 6.8) for the measurement. For calibration and assignments of signals see Materials and Methods section

RESULTS

Synthesis and characterization of 2-aminobenzoyl-CoA

The chemical synthesis of 2-aminobenzoyl-CoA has not yet been reported. This synthesis was not successful in our hands when various published methods for the synthesis of other coenzyme-A thioesters were attempted. For this reason a large scale enzymatic synthesis had to be devised, which relies on a coenzyme A ligase produced and induced in *Pseudomonas* sp. KB 740$^-$, when grown on 2-aminobenzoate as sole source of organic carbon. This enzyme utilizes several benzoic acid derivatives [3], but prefers 2-aminobenzoate; it catalyzes the following reaction: 2-aminobenzoate + ATP + CoA → 2-aminobenzoyl-CoA + AMP + PP_i.

The overall yield was 60% with respect to 2-aminobenzoate added. For the synthesis of ^{14}C-labelled substrate a smaller volume and a slight (1.2-fold) excess of coenzyme A was used resulting in a virtually complete conversion to the [^{14}]C-CoA thioester. The purified thioester was free of contaminants as judged by HPLC and by ^{1}H-NMR spectroscopy. Its ultraviolet/visible spectrum is shown in Fig. 1. The extinction coefficients ($\varepsilon_{355} \approx 5.8\ mM^{-1} \cdot cm^{-1}$ and $\varepsilon_{260} \approx 18.5\ mM^{-1} \cdot cm^{-1}$ at pH 7) were determined using the specific activity of 2-amino[*carboxy*-^{14}C]benzoate. It is worth noting the large bathochromic shift of ≈ 45 nm, which compares to ≈ 65 nm in the case of the anion of 2-hydroxybenzoyl-CoA [10] and indicates a high degree of delocalisation of electrons into the thioester function. The pK_a of ≈ 1.9 is lowered from 4.9 in 2-aminobenzoic acid acid [11] and again reflects the strong withdrawing effect of the thioester function.

The ^{1}H-NMR spectrum (Fig. 2B) is consistent with the structure of 2-aminobenzoyl-CoA and shows, when compared with that of CoA-SH (Fig. 2A), four additional groups of signals (y_1-y_4) originating from the four aromatic protons of the 2-aminobenzoyl moiety. These are partially shifted somewhat upfield (up to $\Delta\delta = -0.13$ ppm) as compared to 2-aminobenzoic acid. The signals of the adenosyl moiety (Fig. 2, l, m and n) are also displaced upfield, suggesting an intramolecular interaction between the 2-aminobenzoyl and the adenine moieties, as this has been observed for other aromatic CoA thioesters [6] and also for FAD [12]. The large downfield shift of the CH_2-S signal (Fig. 2, c) from 2.58 ppm in the CoA-SH spectrum (Fig. 2A) to 3.08 ppm in the 2-aminobenzoyl-CoA spectrum (Fig. 2B) has been reported to be typical for aromatic CoA derivatives such as benzoyl-CoA ($\Delta\delta = +0.50$ ppm) [6].

Analysis of the 2-aminobenzoyl-CoA monooxygenase/reductase-catalyzed reaction by ultraviolet/visible and ^{1}H-NMR spectroscopy and HPLC separation of substrates and products

The reaction catalyzed by this enzyme was routinely followed spectroscopically at 365 nm, where both NADH oxidation and 2-aminobenzoyl-CoA conversion lead to an absorbance decrease [1]. A typical experiment is shown in Fig. 3A where the reaction was started by addition of 2 mol NADH/mol 2-aminobenzoyl-CoA, under non-limiting concentrations of O_2. The appearance of a new maximum at 320 nm and of a shoulder at around 375 nm indicates that a drastic chemical change of the chromophore has occurred; this information is, however, not sufficient for structural interpretations. In order to suppress the contribution of NAD(H) in this spectral region, as well as interferences in ^{1}H-NMR measurements, a system was devised in which the conversion

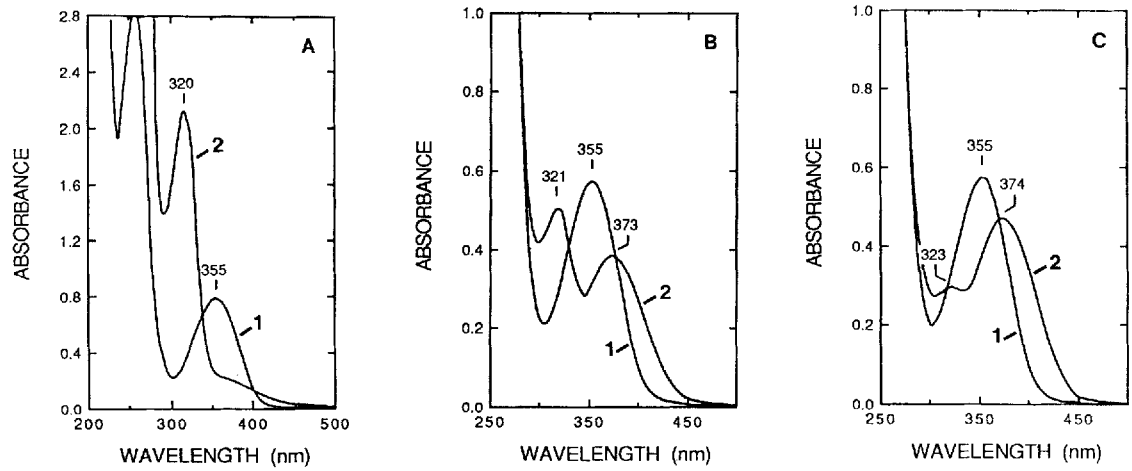


Fig. 3. *Ultraviolet/visible absorption spectra of 2-aminobenzoyl-CoA monooxygenase/reductase incubations before (1) and after completion of the specific reaction (2) carried out using different amounts of NADH.* (A) [NADH]/[2-aminobenzoyl-CoA] = 2:1. (B and C) [NADH]/[2-aminobenzoyl-CoA] = 0.05:1 and 0.02:1, respectively, in the presence of an NADH regenerating system. All incubations contained 0.1 mM 2-aminobenzoyl-CoA in phosphate buffer pH 7.8. For details see Materials and Methods

proceeded in the presence of catalytic amounts of NAD(H) and of an enzymatic regenerating system consisting of formate and formate dehydrogenase. Surprisingly, under these conditions ([NADH]/[2-aminobenzoyl-CoA] = 0.05) the spectral changes during the reaction was substantially different from that in Fig. 3A, as shown in Fig. 3B. From this figure it is apparent that a new chromophore with a $\lambda_{max} \approx 375$ nm was formed in addition to the one giving rise to the 320 nm absorbance. Furthermore, the relative amounts of the products absorbing at the two wavelengths were found to vary depending on the [NAD(H)]/[2-aminobenzoyl-CoA] ratio. This is a first indication, that the shoulder observed around 375 nm in the experiment, in which [NADH]/[2-aminobenzoyl-CoA] ≈ 2, might be due to formation of a mixture of products. Thus, when [NADH]/[2-aminobenzoyl-CoA] was lowered further to 0.02 the absorbance around 375 nm was predominant in the product as shown in Fig. 3C. Analysis of the reaction progress by HPLC showed a new peak at shorter retention time than 2-aminobenzoyl-CoA. When [NADH]/[2-aminobenzoyl-CoA] ≈ 2, this peak, however, was characterized by the presence of a shoulder at longer retention time. The intensity of peak and shoulder was also found to vary depending on the concentration of NADH in the incubation. Thus, under limiting NADH concentrations ([NADH]/[2-aminobenzoyl-CoA] = 0.02) the longer retention time shoulder became the main peak and the main peak became a shoulder. Unfortunately, we did not succeed in completely separating these compounds on a preparative scale. When the reaction products were treated with borohydride, a separation by HPLC in two peaks could be achieved. The minor peak contained the compound absorbing at 375 nm, the major peak exhibited an absorption maximum at 320 nm but cochromatographed slightly different from the original peak. The latter compound was less acid-sensitive.

The ^{1}H-NMR spectrum of the mixture of products obtained at high [NADH]/[2-aminobenzoyl-CoA] is shown in Fig. 4. The salient points emerging are the lack of signals of sufficient intensity in the 'aromatic region', which would have to be present if quantitative conversion to an aromatic compound had occurred. When compared to the spectrum of 2-aminobenzoyl-CoA, the aliphatic region of the spectrum shows the new signals x_1 and x_1, which integrate to at least two protons and which must be assigned to a non-aromatic main product. The position of the CH_2-S signal (Fig. 4, c) is diagnostic for the nature of the activated acid [6]. In Fig. 4, between 2.7 ppm and 3.2 ppm, three signals are apparent, which can be assigned as follows: c_3 has a chemical shift typical for aromatic CoA esters, it is therefore attributed to the new aromatic product; c_2 has a value typical for aliphatic CoA esters, suggesting the presence of a 'reduced' benzoic acid moiety; c_1 can be attributed to $(CoA\text{-}S)_2$ from its chemical shift and might arise from decay of a primary product (cf. below).

The signals at z in Fig. 4, originating from aromatic protons in the 6.5–8-ppm region, clearly cannot be attributed to 2-aminobenzoyl-CoA (compare Figs 2B and 4); they thus must belong to a new aromatic group. An assessment of whether there are 3 or 4 aromatic protons is only possible in a qualitative basis and suggests three protons, which would imply removal of one hydrogen from 2-aminobenzoyl-CoA. Also a qualitative estimate of the integrals of the c signals (Fig. 4) indicates a ratio of $\approx 60\%$, $\approx 20\%$ and $\approx 20\%$ for the aliphatic, the aromatic products and for oxidized CoA, respectively. From these results it is probable that the chromophore having the absorption in the 375 nm region (Fig. 3A) is an aromatic compound.

Stoichiometry of the reaction

Earlier studies of the reaction were carried out using a partially purified enzyme under aerobic conditions [2] and yielded a stoichiometry of 2.7–3.0 NADH oxidized/2-aminobenzoyl-CoA metabolized, concomitantly with production of one equivalent of NH_3. A reinvestigation of the reaction using enzyme purified to apparent homogeneity was carried out under limiting amounts of oxygen and excess of 2-aminobenzoyl-CoA. Under these conditions the consumption of 2-aminobenzoyl-CoA was proportional to that of O_2 as shown by the data in Table 1. The newly determined oxygen/substrate stoichiometry was 0.9–1.0, while the stoichiometry of NADH oxidation/2-aminobenzoyl-CoA consumption was reduced to 2.1–2.4. Furthermore, no ammonia was released. The latter finding, and the apparent discrepancy in comparison with the earlier results, suggests that a hydro-

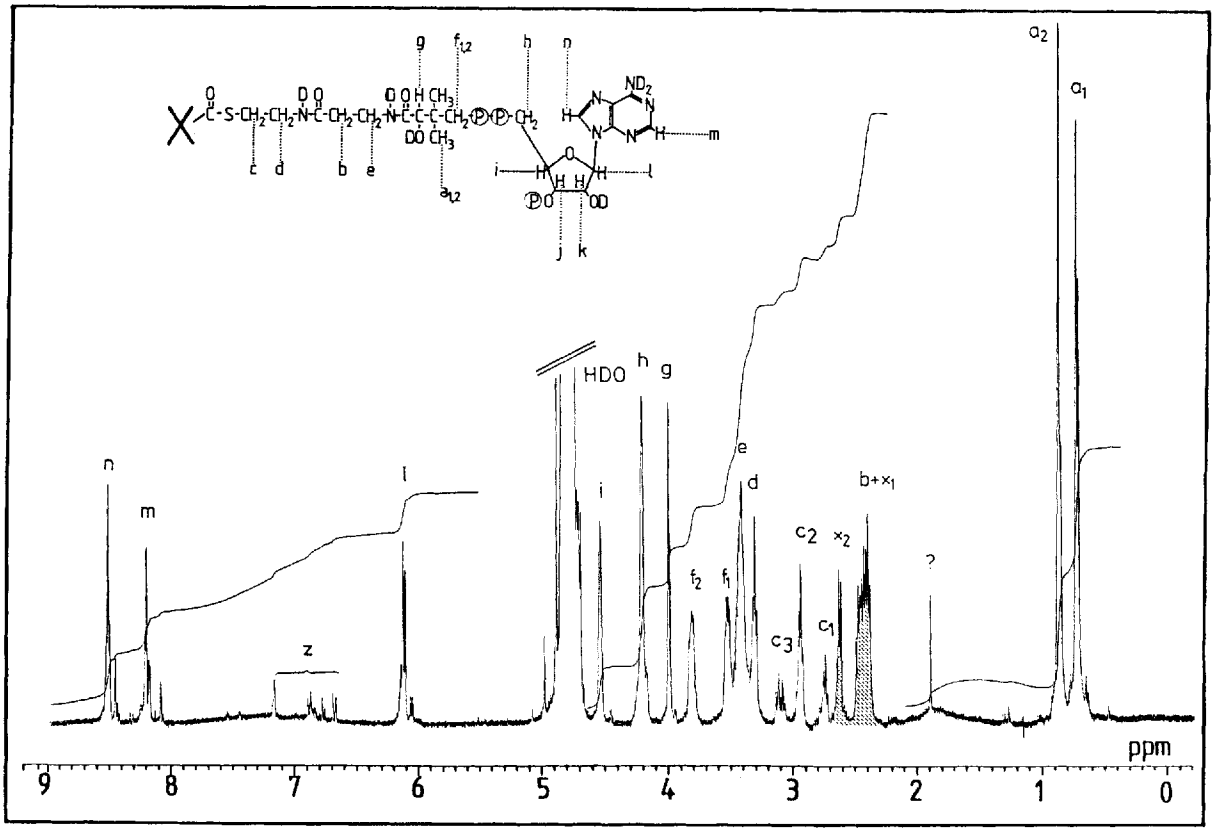


Fig. 4. *^{1}H-NMR spectrum of the product(s) formed from 2-aminobenzoyl-CoA in the 2-aminobenzoyl-CoA monooxygenase/reductase reaction.* The reaction products were isolated from a large scale incubation (1.5 l) by adsorption chromatography and preparative HPLC. The products were lyophilized and redissolved twice in D_2O (pD ≈ 8.7) to yield an approximately 10 mM solution for the measurement. For calibration and assignment see Materials and Methods

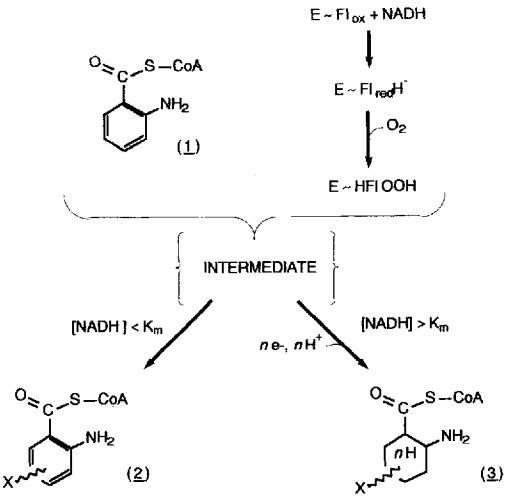
Table 1. *Stoichiometry of the NADH- and oxygen-dependent 2-aminobenzoyl-CoA conversion to coenzyme A thioester(s) of undetermined structure as catalyzed by 2-aminobenzoyl-CoA monooxygenase/reductase*
The diffusion of oxygen into the stoppered cuvet and from the head space into the solution could be neglected during the reaction time. For an exact description of the conditions see Materials and Methods

$[O_2]$ in assay mixture	2-Aminobenzoyl consumed	NADH consumed	Molar ratio of		
			oxidized NADH/O_2	2-Amino-benzoyl-CoA/O_2	NADH/2-amino-benzoyl-CoA
(nmol)					
0	0	0	–	–	–
16	16	37	2.4	1.0	2.4
32	30	64	2.0	0.9	2.1

lytic or a deaminating activity was still present in the former crude enzyme preparation. It also should be pointed out that the stoichiometric value of 2.1 – 2.4 is most probably not due to an experimental error, but probably reflects peculiarities of the reaction as will be discussed below.

DISCUSSION

2-Aminobenzoate is an important intermediate in tryptophan biosynthesis and degradation. While metabolic pathways involving the free acid have been described [13, 15], nothing was known to date on the metabolism of 2-aminobenzoate *via* the coenzyme-A thioester. The results described above demonstrate that the NAD(P)H- and oxygen-dependent conversion of 2-aminobenzoyl-CoA catalyzed by purified 2-aminobenzoyl-CoA monooxygenase/reductase is not a straightforward reaction. At least two types of products (2, and 3, Scheme 1) are formed. Product 2 is not an intermediate in the formation of compound 3, since it is not further metabolized by the enzyme (unpublished results). An important point is the observation of a dependence of the product distribution from the relative NADH concentration. At first sight, it might be assumed to result from a diaphorase activity of the enzyme. This, however, appears unlikely since diaphorase activity was not observed and both the NADH stoichiometry and the product distribution varied depending



Scheme 1. *Proposed reaction sequences for the conversion of 2-aminobenzoyl-CoA catalyzed by the monooxygenase/reductase.* Enzyme-bound oxidized flavin (E ~ Fl_{ox}) is reduced in a first step by one equivalent of NADH resulting in E ~ $Fl_{red}H^-$ (+ NAD^+), and this reacts with molecular oxygen to form the flavin 4a-hydroperoxide (E ~ HFlOOH). This is the species which transfers one atom of oxygen to a not yet specified position of the aromatic nucleus of 2-aminobenzoyl-CoA (1) to form an intermediate, the structure of which is also still open. At low concentrations of NADH, this intermediate will not be reduced and will thus rearomatize to yield a hydroxylated derivative of 2-aminobenzoyl-CoA (2), ([NADH] < K_m). At [NADH] > K_m the intermediate can undergo reduction by one (or two) equivalents of NADH to yield a non-aromatic CoA thioester (3), which is supposed to be the physiological product in the catabolism of 2-aminobenzoic acid by *Pseudomonas* sp. KB 740[7]

on the NADH concentration. The most likely interpretation is that the flavoenzyme has two distinct functions: (a) the requirement of oxygen suggests that the enzyme is of the external monooxygenase type [4], requiring one equivalent of NADH for reducing the flavin coenzyme, which in turn activates oxygen to form an active hydroperoxide [4]; (b) the second activity might consist of the transfer of reducing equivalents to some acceptor other than oxygen, which in this case might be an intermediate occurring during the first catalytic steps of 2-aminobenzoyl-CoA turnover. The presence of a second, reducing activity is supported by the oxygen-independent *N*-ethylmaleimide reduction catalyzed by this enzyme [1].

The ultraviolet/visible spectra and the NMR data suggest that the aromatic nucleus of 2-aminobenzoyl-CoA (1) is still aromatic in the major product (2) formed at low NADH concentrations. The bathochromic shift of $\approx$ 20 nm observed during conversion of compound 1 to this product indicates the introduction of a second functional group interacting with the existing chromophore. ^{1}H-NMR spectra are compatible with the presence of three aromatic hydrogens, which, however, do not correspond to those of 2-aminobenzoyl-CoA. These spectral properties and the type of reaction involved therefore suggests a hydroxylation of the aromatic nucleus (Scheme 1, compound 2, X = OH). Hydroxylation of 2-aminobenzoic acid by a flavin-dependent hydroxylase has been reported to occur at position 3, i.e. ortho to the amino function[16]. The present NMR data do not allow conclusions as to the position of hydroxylation.

At high NADH concentrations ([NADH] > K_m), 2-aminobenzoyl-CoA seems to be converted mainly (80–90%) to a coenzyme-A thioester in which the benzoic acid ring has lost its aromaticity (3). Accordingly, the ^{1}H-NMR spectra (Fig. 4) show the presence of 10–20% of product 2 (compare with Fig. 2B). Concomitantly, new signals are observed in the aliphatic region, integrating to about two protons, which are absent in the spectrum of the starting material (cf. Fig. 2B). Moreover, no evidence for signals of olefinic protons can be found. If the amount of NADH in excess of one equivalent serves in the reduction of the substrate or of an intermediate, then 5–7 new signals should appear in the ^{1}H-NMR spectra of the product. This apparent discrepancy is probably the result of ^{1}H/^{2}H exchange, which in turn suggests that oxo/enol equilibria must be possible. This is in agreement with the observed reactivity of the product with borohydride and suggests the presence of either an imino or of a oxo function. The ultraviolet absorption ($\lambda_{max} \approx 320$ nm) is compatible with the presence of an 2,3-unsaturated thioester which, however, is different from 3-oxo-acyl-CoA (λ_{max} = 300 nm, ($\varepsilon \approx 18\ mM^{-1} \cdot cm^{-1}$ at pH 9.0 [17]). Furthermore, the ultraviolet spectra also exclude reduction to a completely saturated species since the latter have absorption maxima below 270 nm [17].

In conclusion, our results are compatible with a branching mechanism such as that shown in Scheme 1. Here the (oxidized) flavoenzyme is first reduced by NADH and reacts subsequently to form an enzyme hydroperoxide, as has been generally proposed for this type of hydroxylase [4]. This species does not appear to decay unproductively to oxidized enzyme and H_2O_2 in the presence of the aromatic substrate. The subsequent reaction involves transfer of (probably) one oxygen to the aromatic group to form an intermediate, which must have a finite life-time since its further reaction partitions between two pathways depending on the concentration of NADH: The first reaction, occurring in the absence of NADH, is proposed to consist in the rearrangement of the intermediate to yield a hydroxylated aromatic compound 2; whether this occurs on the enzyme or upon dissociation cannot be decided. In the presence of NADH, either the flavin is reduced first then it transfers reducing equivalents to the (still bound) intermediate, or reduction of the latter occurs directly from NADH. Dissociation of the intermediate from the enzyme and subsequent non-enzymatic reduction by free NADH cannot be excluded at present; however, it appears to be unlikely since high concentrations of NADH would probably be required to favour exclusive formation of the reduced product 3. In a forthcoming paper we will report on ongoing experiments aimed at the elucidation of the chemical structure of these product(s).

REFERENCES

1. Buder, R. & Fuchs, G. (1989) *Eur. J. Biochem 185*, 629–635.
2. Ziegler, K., Braun, K., Böckler, A. & Fuchs, G. (1987) *Arch. Microbiol. 149*, 62–69.
3. Ziegler, K., Buder, K., Winter, J. & Fuchs, G. (1989) *Arch. Microbiol. 151*, 171–176.
4. Ghisla, S. & Massey, V. (1989) *Eur. J. Biochem. 181*, 1–17.
5. Braun, K. & Gibson, D. T. (1984) *Appl. Environ. Microbiol. 48*, 102–107.
6. Mieyal, J. J., Webster, L. T. Jr. & Siddiqui, U. A. (1973) *J. Biol. Chem. 249*, 2633–2640.
7. Lee, C.-H. & Sarma, R. H.J. (1975) *J. Am. Chem. Soc. 97*, 1225–1236.
8. Munsche, D. & Schütte, H. R. (1963) *Z. Chem. 6*, 230–231
9. Da fonseca-Wollheim, F., Bergmeyer, H. U. & Gutmann, I. (1974) in *Methoden der enzymatischen Analyse* (Bergmeyer, H.U., ed) pp. 1850–1853, Verlag Chemie, Weinheim.

10. Webster, L. T. Jr., Mieyal, J. J. & Siddiqui, U. A. (1973) *J. Biol. Chem. 249*, 2641–2645.
11. Dawson, R. M. C., Elliott, D. C., Elliott, W. H. & Jones, K. M. (1986) in *Data for biochemical research*, p 6 , Clarendon Press, Oxford.
12. Müller, F., Ghisla, S., & Bacher, A., (1988) in *Vitamine II* (Isler, O., Brubacher, G., Ghisla, S. & Kräutler, B., eds) p 88, G. Thieme, Stuttgart, New York.
13. Cain, R. B. (1968) *Antonie van Leeuwenhoek 34*, 417–432.
14. Anderson, J. J. & Dagley, S.(1981) *J. Bacteriol. 146*, 291–297.
15. Kobayashi, S., Kuno, S. & Itada, N. (1964) *Biochim. Biophys. Res. Commun. 16*, 556–561.
16. Powlowski, J. B., Dagley, S., Massey, V. & Ballou, D. P. (1987) *J. Biol. Chem. 262*, 69–74.
17. Decker, K. (1959) in *Die aktivierte Essigsäure* (Pummerer, R., ed.) pp 75–87, Enke Verlag, Stuttgart.