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

Identification of the reaction products

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In a previous report we have described some properties of a novel flavoenzyme from a denitrifying *Pseudomonas* species which catalyzes the oxygen- and NAD(P)H-dependent conversion of 2-aminobenzoyl-CoA [Buder, R., Ziegler, K., Fuchs, G., Langkau, B. & Ghisla, S. (1989) *Eur. J. Biochem. 185*, 637–634]. In this paper, we report on the identification of the three products formed from 2-aminobenzoyl-CoA in this reaction. The spectroscopic data and the chemical properties of these compounds and those of their degradation products are compatible with the structures of 2-amino-5-hydroxybenzoyl-CoA, 2-amino-5-hydroxycyclohex-1-enecarboxyl-CoA and of 2-amino-5-oxocyclohex-1-enecarboxyl-CoA. The latter is the main product and was found to be rather unstable since it hydrolyzes and decarboxylates readily at pH < 5. Ammonia is released from the decarboxylation product in the neutral pH range to yield 1,4-cyclohexanedione. Conditions were optimized such that the CoA thioester of 2-amino-5-hydroxybenzoate is the product obtained at > 98% yield. 2-amino-5-hydroxycyclohex-1-enecarboxyl-CoA is the product which is formed when the mixture of the reaction products is treated with sodium borohydride before separation.

A new flavoenzyme was recently discovered which appears to play a key role in the aerobic metabolism of 2-aminobenzoic acid (anthranilic acid) in a denitrifying *Pseudomonas* species [1–4]. This enzyme is quite unusual since it appears to catalyze monooxygenation as well as hydrogenation of the substrate; therefore it has tentatively been named 2-aminobenzoyl-CoA monooxygenase/reductase [5]. In a previous paper we have investigated the stoichiometry and some aspects of this reaction [2]. In an oxygen- and NAD(P)H-dependent reaction at least two products were formed from 2-aminobenzoyl-CoA, depending on the NADH concentration. At $[NADH] < K_m$, a CoA thioester was almost exclusively formed which is characterized by a $\lambda_{max} = 379$ nm at pH 7. This product retained the aromatic character and was not metabolized further. At $[NADH] > K_m$, a product was formed predominantly which is characterized by a $\lambda_{max} = 320$ nm at pH 7. This probably non-aromatic species is assumed to be the physiological product [2]. Formation of a third product in minor and varying quantities was also observed, showing a similar absorption spectrum to that of the latter product ($\lambda_{max} = 320$ nm).

In this paper we have studied the chemical structure of these products using ^{1}H-NMR, ultraviolet/visible and fluorescence spectroscopy and various chromatographic techniques. This study provides the first insights into a novel type of enzymatic reaction in which the same flavin cofactor is involved both in a hydroxylation and a hydrogenation reaction. The nature of the products allows some conclusions about the mechanism of monooxygenation by flavin-dependent hydroxylases.

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Enzymes. 2-Aminobenzoyl-CoA monooxygenase/reductase (EC 1.14.13.–); cyclohexanol dehydrogenase (EC 1.1.1.–); formate dehydrogenase (EC 1.2.1.2); glutamate dehydrogenase (EC 1.4.1.3).

MATERIALS AND METHODS

Chemicals and biochemicals

The biochemicals and the enzymes formate dehydrogenase (from yeast) and glutamate dehydrogenase (from beef liver) were from Boehringer (Mannheim, FRG). [^{14}C]carboxylate-labelled and unlabelled 2-aminobenzoyl-CoA were synthesized and purified as described in a preceding paper [2]. Phthalaldehyde, 1,4-cyclohexanedione, 2-amino-5-hydroxybenzoic acid and other reference substances were from Fluka (Buchs, Switzerland); 1,4-cyclohexanediol from Merck-Schuchardt (München, FRG). Materials for GLC were obtained from Macherey and Nagel (Düren, FRG).

Enzyme assays and determinations

The activity of 2-aminobenzoyl-CoA monooxygenase/reductase was followed spectrophotometrically at 365 nm [1]. The cyclohexanol dehydrogenase assay was performed as described earlier [6]. This set up was also employed for the identification of 1,4-cyclohexanedione, via conversion to 4-hydroxycyclohexanone and 1,4-cyclohexanediol. NH_3 was determined enzymatically [7].

Instrumentation

^{1}H-NMR spectra were recorded with a JEOL GX 400 (400 MHz) spectrometer at 25°C. The HDO signals were suppressed by common irradiation techniques. For calibration of ^{1}H, the *g* signal of CoA esters (4.00 ppm) was used as an internal standard [2, 8]. The assignment of signals of the CoA moiety is described elsewhere [9]. For the ultraviolet spectroscopy a Kontron UVIKON 810 instrument was used. Fluorescence spectra were obtained with a Perkin-Elmer MPF-4 fluorimeter. HPLC analyses were carried out using different reversed-phase columns (see below) and detection with either

a photodiode array (Merck/Hitachi) or a spectromonitor D (LDC/Milton Roy) in combination with a F1000 fluorescence spectrometer (Merck/Hitachi). GLC analyses were performed with a Carlo Erba gas chromatograph equipped with a flame-ionization detector and an integrator (Merck/Hitachi). Nitrogen was used as the carrier gas at a flow rate of 40 ml/min. TLC analyses were carried out on 0.25 mm Kieselgel G and aluminum oxide Type E (E. Merck, Darmstadt, FRG) with chloroform or ethyl acetate as solvents.

*Enzymatic conversion of 2-aminobenzoyl-CoA
under limiting NADH concentration and purification
of 2-amino-5-hydroxybenzoyl-CoA*

An incubation mixture containing 2.0 μmol 2-aminoben-zoyl-CoA, 0.02 μmol NADH, 0.04 μmol FAD, 10.0 μmol formate, and 0.1 μmol pyrazole in 20 ml 100 mM potassium phosphate buffer, pH 7.8, was stirred at 25 °C. The reaction was started by addition of 0.025 units formate dehydrogenase and 0.013 units enzyme. Substrate consumption and product formation were monitored by ultraviolet spectroscopy and HPLC (see below). After 16 h about 60% 2-aminobenzoyl-CoA had reacted. The reaction was stopped by application of the solution containing the products on a Sepralyte-cyclo-hexane column (30 mm × 9 mm, ICT, Frankfurt, FRG) equilibrated with 50 mM potassium phosphate buffer, pH 7.8. Using this method, 2-amino-5-hydroxybenzoyl-CoA was separated from all hydrolysis products since only CoA thioesters adsorbed and subsequently eluted with water. Separation from unreacted substrate, 2-aminobenzoyl-CoA, was achieved by preparative HPLC on a RP-8 Spherisorb column (400 mm × 8 mm, Knauer, Freiburg, FRG) using 86.5% 50 mM potassium phosphate buffer, pH 7.8, 6.5% acetonitrile and 7.0% methanol as solvent.

Hydrolysis of 2-amino-5-hydroxybenzoyl-CoA

For esterolysis the product was incubated in NaOH, pH 12.2, at 37 °C for 12 h. The reaction mixture was adjusted to pH 6.0 with 1 M HCl and the products were separated on a half-preparative RP-18 Spherisorb column (250 mm × 8 mm, Bischoff, Leonberg, FRG) at a flow rate of 2 ml/min using the same solvent system as for analysis of CoA thioesters and their hydrolysed products on analyticyl HPLC (see below).

Analysis of hydroxylated aromatic products by HPLC

For analytical HPLC of 2-amino-5-hydroxybenzoyl-CoA and the hydrolysed products we used a RP-18 LiChrosorb column (250 mm × 4 mm, Merck, Darmstadt, FRG) and the following solvent systems at a flow rate of 1 ml/min. Solvent A: 20 mM sodium phosphate buffer, pH 6.0. Solvent B: 95% aqueous methanol. For analysis of CoA and CoA thioesters a gradient of 10−45% (by vol.) solvent B in solvent A was used. Analysis of 2-amino-5-hydroxybenzoate was performed isocratically using either solvent A or solvent A containing 5% or 10% solvent B.

*Reaction of 2-aminobenzoyl-CoA
at non-limiting NADH concentration, hydrolysis,
and analysis of products*

A solution (1 ml in a spectral cuvette) of 0.6−1.0 μmol 2-aminobenzoyl-CoA was reacted in the presence of purified enzyme under normal enzyme assay conditions [1]. Due to

substrate inhibition at concentrations of 2-aminobenzoyl-CoA > 200 μM, the latter was added in 0.2-μmol portions and NADH in 0.7-μmol portions. In order to ensure a sufficient supply of oxygen the cuvette was shaken gently by hand at regular intervals over a period of approximately 30 min. The progress of the reaction was monitored spectrophotometrically at 365 nm. Hydrolysis of the thioester product mixture was performed by adjusting the pH to 0.8 with 6 M HCl. In order to measure the release of ammonia enzymatically [7], the hydrolysis products were incubated at pH 8.5 (adjustment of the pH with 6 M NaOH) for 5 min at ambient temperature. For GLC analyses the products were reacidified with 6 M HCl (to pH 0.8) and analyzed either by direct injection of the acidic solution on a 2 mm × 1.80 m GLC glass column packed with 10% SP1000 in 1% H_3PO_4 on Chromosorb WAW (80/100 mesh), or by extraction in an equal volume of chloroform and injection onto a 5% Carbowax (20 M) on Chromosorb WAW (80/100 mesh) column. Following TLC, the main hydrolysis product was detected by spraying with 0.4% 2,4-dinitrophenol in 2 M HCl. For the fluorimetric determination of 1,4-cyclohexanedione a 100-μl sample was taken from the incubation under the conditions described in [10], and added to 900 μl 0.2% phthalaldehyde in concentrated sulfuric acid, and analyzed as described by Sawicki et al. [10].

1,4-Cyclohexanedione, 1,4-cyclohexanediol and 4-hydroxycyclohexanone were identified and quantified by GLC comparison with authentic samples. 4-Hydroxycyclohexanone was prepared enzymatically from 1,4-cyclohexanedione and NADH using extracts from a strain of *Pseudomonas* species which was grown anaerobically on cyclohexanol and which contained cyclohexanol dehydrogenase activity (800 nmol · min^{-1} · mg^{-1} protein) [6]. Alternatively, 4-hydroxycyclohexanone was synthesized chemically according to [11].

*Preparative incubation of 2-aminobenzoyl-CoA
at non-limiting NADH concentration, treatment
with borohydride and purification*

$NaBH_4$ reduction of the primary reaction product(s) upon incubation with excess NADH and subsequent isolation of the (stabilized) main product by preparative HPLC were described in a previous paper [2]. For analytical HPLC an RP-18 LiChrosorb column, 25 cm × 0.4 cm was employed, using 90% (by vol.) 50 mM phosphate buffer, pH 6.7, 7% (by vol.) acetonitrile and 3% (by vol.) methanol as mobile phase.

Decarboxylation of reaction products

The decarboxylation of the reaction products of 2-aminobenzoyl-CoA monooxygenase/reductase was followed by determining the loss of ^{14}C from the solution upon flushing with 20% CO_2/80% N_2 (by vol.). 2-Amino-[carboxy-^{14}C]-benzoyl-CoA was used as substrate. Radioactivity was measured with a scintillation counter from Kontron.

RESULTS AND DISCUSSION

*Isolation and identification of 2-amino-5-hydroxybenzoyl-CoA,
the main product formed under NADH-limiting conditions*

For the production of substantial amounts of this compound, the conditions described previously [2], which essentially involved the use of a NADH-regeneration system, were
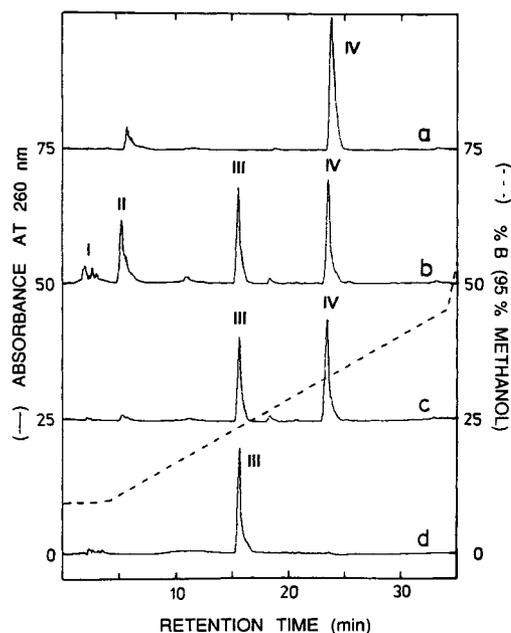
Fig. 1. *HPLC analysis of course of formation and of purification of 2-amino-5-hydroxybenzoyl-CoA (cf. Cheme 1, 5).* (a) 2-Aminobenzoyl-CoA (peak IV), used for the reaction; note traces of hydrolysis products anthranilic acid and CoA-SH (peak II). (b) Reaction mixture obtained upon incubation under NADH-limiting conditions. Peak III is the product 2-amino-5-hydroxybenzoyl-CoA and peak I, NAD$^+$/NADH. (c) Analysis of the products obtained upon separation from NAD(H) (I), CoA-SH (II) and anthranilic acid (II) by Sepralyte-cyclohexane adsorbance. (d) Pure 2-amino-5-hydroxybenzoyl-CoA (III)

adapted to a larger scale and the [NADH]/[2-aminobenzoyl-CoA] ratio was lowered substantially to 0.01 as described in Materials and Methods. Under these conditions this product (III) was formed predominantly from 2-aminobenzoyl-CoA (IV), as shown in Fig. 1a and b. It should be noted, that complete conversion of 2-aminobenzoyl-CoA was not achieved under these conditions; the reaction was stopped afer $\approx$ 30 h when a reasonable compromise between product formation ($\approx$ 40%) and hydrolysis of thioesters ($\approx$ 20%) was observed. The chromatogram in Fig. 1c is that of the mixture obtained upon separation on a Sepralyte-cyclohexane column, and demonstrates the efficient and easy separation from NAD(H) (I), CoASH (II) and 2-aminobenzoate (II). The chromatogram in Fig. 1d documents that product (III) elutes as a single peak (fluorescence and ultraviolet detection) upon preparative HPLC purification.

Its absorption spectrum is markedly dependent on the pH, as shown in Fig. 2, and the changes reflect two ionisations with pK_a values of 3.2 and 10.2. The shift of the λ_{max} from 355 nm in 2-aminobenzoyl-CoA to 379 nm (at neutral pH values) in the product suggest that the chromophore has been elongated by introduction of a new function. The pK_a of 3.2 (Fig. 2A) is attributed to the amino function in position 2. The second pK_a of 10.2 (Fig. 2B) is compatible with the presence of a phenolic function, which might be located at positions 3, 4, 5, or 6 of the benzene moiety. This is compatible with the disappearance of the lowest energy transition at low pH (protonation of the amino function) and with the bathochromic shift of the same band at high pH (deprotonation of aromatic OH group). Information about the location of the new function is provided by the ^{1}H-NMR spectra shown in Fig. 3. The presence of three signals each integrating to one hydrogen in
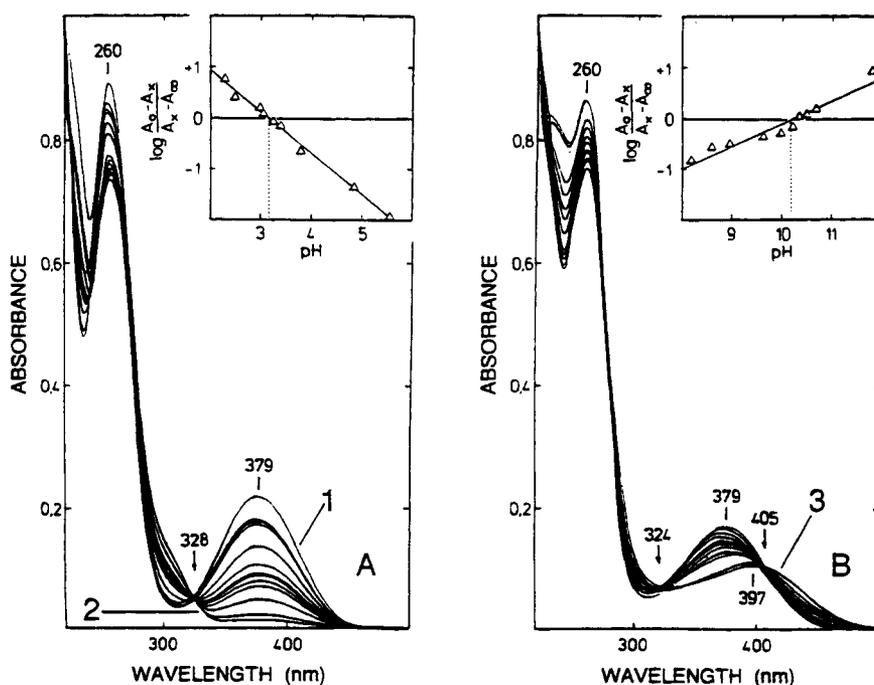

Fig. 2. *pH Dependence of the ultraviolet/visible absorption spectrum of 2-amino-5-hydroxybenzoyl-CoA isolated as the main product from the NADH-limiting conversion of 2-aminobenzoyl-CoA.* Spectrum (1) was obtained in 50 mM phosphate buffer, pH 7.8 and subsequent traces upon addition of small amounts of first (A) 0.1 M HCl then (B) 0.1 M NaOH. The absorption changes obtained at the pH values measured are plotted in the inserts. Curves (2) and (3) were obtained at pH 1.9 and pH 12.7. Isosbestic points are at 328 nm in (A), and at 324 nm and 405 nm in (B)
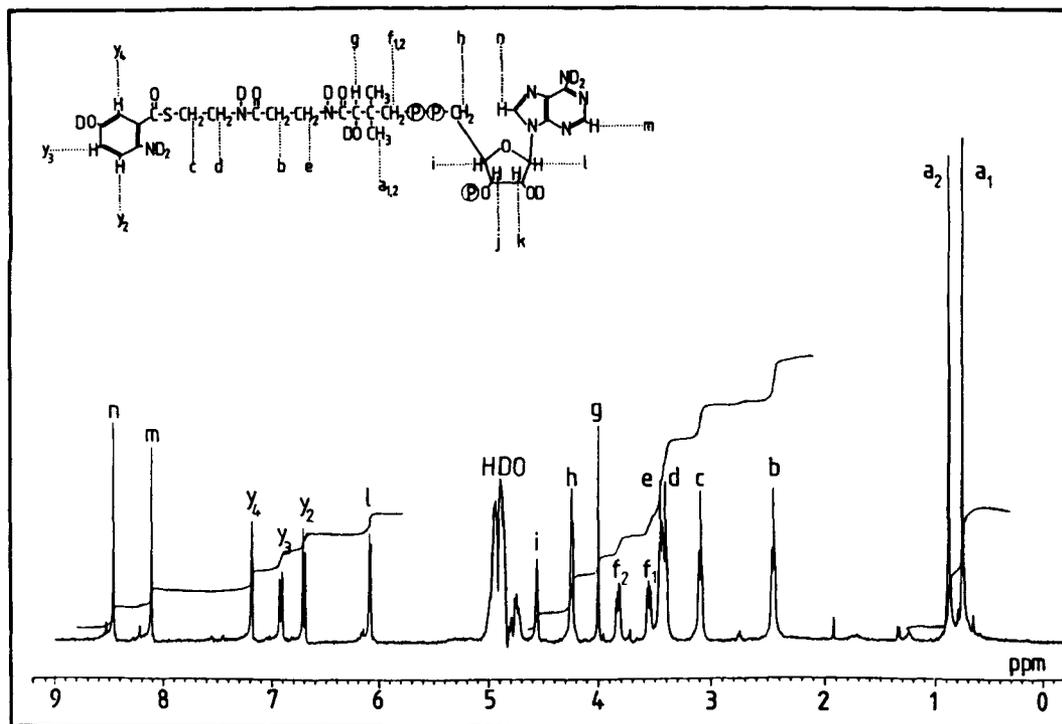
Fig. 3. ^1H-NMR spectra of 2-amino-5-hydroxybenzoyl-CoA, the main product obtained under NADH-limiting conditions. A purified sample (0.5 ml, 11 mM) was lyophilized and redissolved in 0.5 ml D_2O (pD $\approx$ 8.5). For calibration and assignments, see Materials and Methods

the range 6.7 – 7.2 ppm confirms the removal of one hydrogen in the ring, and the reciprocal couplings of these signals (two doublets and a singlet) exclude positions 3 and 6 as the point of substitution. The relative chemical shifts of the three signals suggest that the new substituent is at position 5 rather than 4; the latter, however, cannot be excluded by ^1H NMR.

Final proof of the 2-amino-5-hydroxybenzoyl-CoA structure (see Scheme 1, 5) was obtained by analysis of the product obtained upon alkaline hydrolysis. This product was different from authentic 2-amino-4-hydroxybenzoic acid, but indistinguishable from authentic 2-amino-5-hydroxybenzoic acid (Scheme 1, 12) on HPLC (retention time, ratio of absorbance to fluorescence emission intensities in runs in which both parameters were determined concomitantly), ultraviolet absorption spectra at pH values of 2.0, 6.7 and 11.5 and fluorescence excitation (λ_{max} = 333 nm) and emission spectra (λ_{max} = 430 nm).

*Isolation and identification
of 2-amino-5-oxocyclohex-1-enecarboxyl-CoA, the product
formed at high (physiological) NADH concentration*

This reaction product, which is considered as the physiologically important metabolite, was formed when NADH ($\approx$ 0.5 mM) was present at 3 – 5-fold excess over 2-amino-benzoyl-CoA during the incubation (see Materials and Methods for experimental conditions). On preparative HPLC this main product could not be separated completely from further minor product(s) which, however, were formed only in small amounts under these conditions. The ultraviolet spectrum of this product mixture is similar to that of 2-amino-5-hydroxycyclohex-1-enecarboxyl-CoA, which is shown in Fig. 4, a minor difference to the latter being a shoulder around 380 nm (probably due to the presence of some 2-amino-5-

hydroxybenzoyl-CoA) and to a higher A_{260}/A_{320}. The spectrum of the main product obtained by rapid spectroscopy during HPLC separation (see Materials and Methods for details) has maxima at 260 nm and 320 nm of approximately equal intensity. This chromophore is thus basically different from that of an aromatic 2-aminobenzoyl-CoA derivative, as demonstrated using 2-amino-5-hydroxybenzoyl-CoA (Fig. 2A). This observation and the stoichiometry of > 2 mol NADH/mol 2-aminobenzoyl-CoA determined for its formation suggest that (at least) two reducing equivalents have been taken up by the aromatic moiety of the substrate. One equivalent of NADH is probably required for the reduction of the flavin during the monooxygenation reaction [5]. At pH < 3 the absorbance band centred at 320 nm is lost, however the corresponding pK could only be estimated as 3 – 4 since the spectral changes were only partially reversible. This is attributed to rapid hydrolysis/decarboxylation of this product, and is confirmed by the loss of label as $^{14}CO_2$ from the [^{14}C]carboxylate-labelled product derived from 2-amino-[carboxy-^{14}C]benzoyl-CoA.

The pK of 3 – 4 is probably due to ionisation of an amino or imino group, which should logically be at position 2, since ammonia was not released under the conditions of enzymatic conversion. Aromatic hydroxyl groups should have pK_a values > 8.5 [12] and a value of 3 – 4 appears to be too low for the ionisation of a β-oxothioester since e.g. acetoacetyl-CoA has $pK \approx$ 8.6 [13]. Also, no effect of Mg^{2+} [5 mM] on the spectra or on their pH dependence was observed. Mg^{2+} has been reported to stabilize the enolate form of β-oxoacids [13].

The product obtained (in addition to CoASH) upon acid decarboxylation at pH 0.8 did not absorb in the near ultraviolet/visible region and therefore was not detectable on HPLC. Upon this acidic incubation one equivalent of NH_3 was determined suggesting the presence of an imino or enamine function. The ultraviolet-undetectable product was
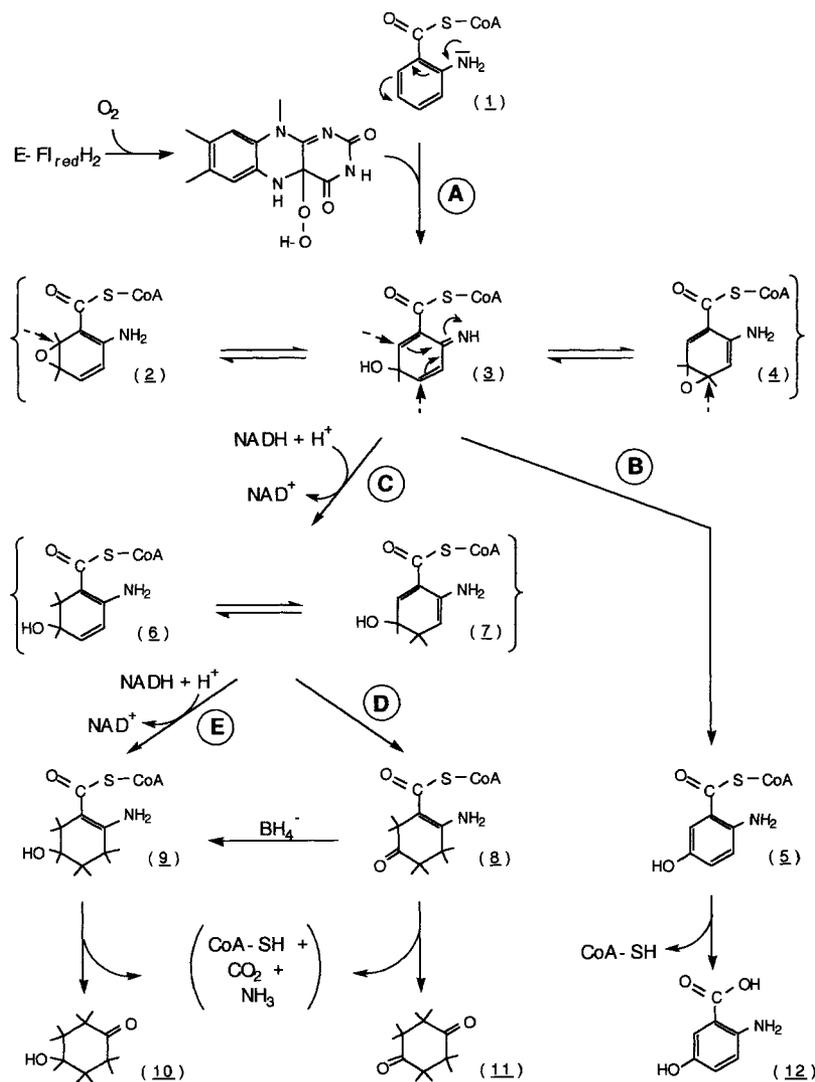
Scheme 1. *Sequences proposed for the reaction catalyzed by 2-aminobenzoyl-CoA monooxygenase/reductase.* The intermediates in parentheses are hypothetical, whereas the chemical structures of compounds 5 and 8 — 12 are consistent with the products identified as described in the paper. Steps A — E are discussed in Results and Discussion
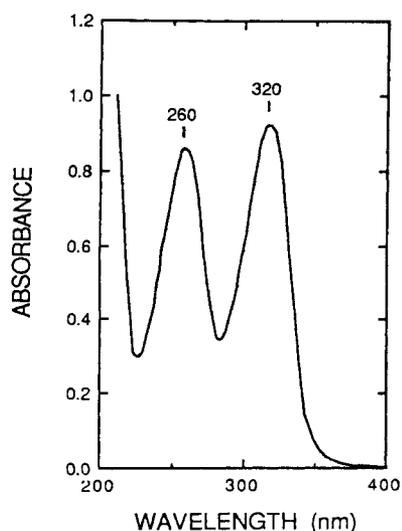

Fig. 4. *Ultraviolet absorption spectrum of 2-amino-5-hydroxycyclohex-1-enecarboxyl-CoA in 50 mM phosphate buffer, pH 7.0.* The spectrum was obtained from the reaction product under non-limiting NADH conditions upon treatment with sodium borohydride and subsequent purification on preparative HPLC

extracted with $CHCl_3$ at pH 0.8, and on TLC and GLC (cf. Table 1) it co-chromatographs with authentic 1,4-cyclohexanedione in different systems. The presumed 1,4-cyclohexanedione was obtained in a 60% yield relative to 2-aminobenzoyl-CoA as the starting material. More than 30% of the theoretically expected 1,4-cyclohexanedione was found by fluorimetric estimation according to the method used in [10]. A second hydrolysis product amounting to approximately 13% of the total in the mixture co-chromatographed on GLC with authentic 4-hydroxycyclohexanone [11]. The same products were obtained when 1,4-cyclohexanedione was incubated with cyclohexanol dehydrogenase and NADH (Table 1).

Treatment of the reaction mixture obtained at high concentrations of NADH with borohydride, and properties of the products

Further information on the structure of the reaction products was obtained from experiments in which the primary reaction mixture obtained at [NADH] ≈ 0.5 mM was treated with sodium borohydride in an attempt to reduce the expected carbonyl or imino functions and to stabilize the components. When such a mixture, which has an absorption maximum at

320 nm and a shoulder around 380 nm, was incubated with approximately 0.5 mg $NaBH_4$/ml sample, at pH 7.9 for 30 min, the double peak eluting on HPLC with retention times of 7.35 min and 7.90 min separated into two peaks (not shown). The chromophore absorbing at $\approx$ 379 nm (i.e. 2-amino-5-hydroxybenzoyl-CoA; Scheme 1, 5) remained essentially unaffected by the borohydride treatment (retention time, 7.49 min), whereas a new (main) band appeared with a somewhat shorter retention time (5.94 min); it has an absorbance, which is closely similar to that of the untreated product (i.e. λ_{max} = 320 nm), as judged by spectra obtained from a diode-array detector. In contrast to the untreated mixture, upon reaction with $NaBH_4$ the main product could thus be separated by HPLC from 2-amino-5-hydroxybenzoyl-CoA.

The absorption spectrum of the purified new product (Fig. 4) does not differ significantly from that of 2-amino-5-oxocyclohex-1-enecarboxyl-CoA (Scheme 1, 8), i.e the product obtained without $NaBH_4$ treatment. This indicates that borohydride does not alter the chromophore associated with the 320-nm absorption band itself, although it does modify the product since it affects its HPLC retention time.

The ^{1}H-NMR spectrum of the purified CoA derivative is shown in Fig. 5. As expected, aromatic hydrogens are absent, while there are new signals $(x_1 - x_6, x_7)$ integrating to approximately seven protons in the aliphatic region. Importantly the signal x_7 at $\approx$ 3.98 ppm corresponds to that of the C(OH)-H in 4-hydroxycyclohexanone.

In toto, the available evidence is compatible with the structure of 2-amino-5-hydroxycyclohex-1-enecarboxyl-CoA (cf. Scheme 1, 9). This suggests that the (main) product of 2-aminobenzoyl-CoA monooxygenase/reductase conversion under non-limiting NADH conditions is 2-amino-5-oxocyclohex-1-enecarboxyl-CoA (8), and that 2-amino-5-hydroxycyclohex-1-enecarboxyl-CoA is formed as a minor component. Borohydride treatment reduces the 5-oxo group in (8) forming (9), which is then identical with the secondary product. These conclusions are also supported by the finding that acid hydrolysis of the borohydride-reduced product yields 4-hydroxycyclohexanone (Scheme 1, 10), rather than 1,4-cyclohexanedione (Scheme 1, 11), as analyzed by GLC (not shown).

Product of the catalytic reaction with N-ethylmaleimide

The observation that the enzyme reduces N-ethylmaleimide in an oxygen-independent reaction consuming NADH is valuable for the understanding of the enzyme reaction mechanism. Following up a previous suggestion [1], we have identified the product of this reaction as succinimide using various TLC systems. The stoichiometry is 1 mol NADH oxidized/mol succinimide consumed in the assay.

Conclusions

The information on the chemical structure of three different products (Scheme 1, products 5, 8 and 9), which are formed in varying quantities depending on the concentration of NADH used in the incubation, provides some insight into the mechanism catalyzed by 2-aminobenzoyl-CoA monooxygenase/reductase. This is the first example of a monooxygenation reaction of an aromatic compound, catalyzed by a flavin enzyme, which does not lead necessarily to an aromatic final product. The formation of the different products 5, 8, and 9 require the occurrence of the two types of intermediates 2, 3,

4, 6, and 7 in Scheme 1. From this, the mechanism of Scheme 1 can be put forward. The first step is the activation of molecular oxygen by the reduced flavin of the monooxygenase to form a flavin 4a-hydroperoxide; this is in accordance with what has been found up to now with all enzymes belonging to this class [5]. This reaction consumes one equivalent of NADH for the generation of reduced falvin (not shown). The subsequent process is envisaged to be the hydroxylation of the aromatic moiety of 2-aminobenzoyl-CoA (1), and is not dependent on the availability of NADH (Scheme 1, step A). This would result in one of the possible, oxygenated intermediates 2, 3 or 4, a required feature being that oxygen must be attached to position 5 of the aromatic compound.

From this point on, a branching must occur, which depends on the presence or absence of further reducing equivalents. In their absence a re-aromatization is seen to occur yielding the hydroxylated non-reduced product 5 via step B. This might occur at the enzyme active centre, or upon dissociation. In the case of availability of reducing equivalents either one of intermediates 2, 3 or 4 can react to yield intermediates 6 or 7. Again, at this stage a similar type of competition between steps D and E is envisaged to occur. Step D, which is a ketonisation occurring either at the active site or upon dissociation, leads to the main product 8. The competing reduction reaction (E) forms 9 from either 6 or 7.

What differentiates the present results from all data published to date on flavin-dependent hydroxylases is the occurrence of intermediate(s) with lifetime(s) of sufficiently long to allow partitioning between different reactions. This is of eminent importance in the context of the mechanism of insertion of activated oxygen into aromatic substrates by flavin monooxygenases since it might allow the elucidation of the structure of the intermediates itself, and thus provide new information on the mechanism of insertion of oxygen into the substrate.

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