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2-Aminobenzoyl-CoA monooxygenase/reductase

Evidence for two distinct loci catalyzing substrate monooxygenation and hydrogenation

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2-Aminobenzoyl-CoA monooxygenase/reductase catalyzes both monooxygenation and hydrogenation of anthraniloyl-CoA. Its reactivity with 11 substrate analogs has been investigated. Only 2-aminobenzoyl-CoA (anthraniloyl-CoA) in its normal and deuterated (5-^{2}H) form is a full substrate, and only traces of 2-hydroxybenzoyl-CoA (salicyloyl-CoA) are probably monooxygenated but not hydrogenated. The purified enzyme is a homodimer and has been resolved preparatively into three major species by anion-exchange chromatography on Mono Q. All three species have the same specific activity when reconstituted to full content of FAD, they differ, however, substantially in their mode of binding FAD. The oxidized or fully reduced enzyme forms bind tightly 0.5 mol/mol of the substrate 2-aminobenzoyl-CoA ($K_d = 1-2$ μM). The enzyme can be depleted of ≈50% of its FAD, which corresponds to essentially complete removal from one of the two binding sites, reflecting a large difference in the affinity for FAD. From this it is deduced that the two sites are not equivalent. Removal of FAD from one binding site leads to loss of the hydrogenation capacity of the enzyme, while monooxygenation catalysis is retained. The FAD cofactors of the two binding sites differ drastically in their reactivities towards NADH, oxygen and *N*-ethylmaleimide. Exchange of reducing equivalents between the two FAD cofactors at the respective binding sites is very slow and irrelevant compared to the rates of catalysis. It is concluded that the enzyme, which has been proposed to consist of two identical polypeptide chains [Altenschmidt, U., Bokranz, M. & Fuchs, G. (1992) *Eur. J. Biochem. 207*, 715–722], contains two active centers which differ substantially in their catalytic activity. One center belongs to the class of monooxygenases, the other one to the (de)hydrogenases. This must result from substantially different interaction of the same flavin cofactors with protein functional groups and is, to our knowledge, unprecedented in flavoprotein enzymology.

Keywords. 2-Aminobenzoyl-CoA; monooxygenase; reductase; flavoprotein; kinetics.

2-Aminobenzoyl-CoA monooxygenase/reductase (ABzCoA-M/R), is a recently discovered novel type of flavoprotein, which, under physiological conditions, catalyzes the two following reactions (Langkau et al., 1990):

$$\text{ABzCoA} + \text{NADH} + \text{H}^+ + \text{O}_2 \rightarrow \text{ABzOHCoA*} + \text{NAD}^+ + \text{H}_2\text{O} \quad (1)$$

$$\text{ABzOHCoA*} + \text{NADH} + \text{H}^+ \rightarrow \text{A(OH)H}_2\text{CoA} + \text{NAD}^+ \quad (2)$$

where ABzCoA is the substrate anthraniloyl-CoA, ABzOH-CoA* is a postulated primary intermediate, the non-aromatic, quinoid tautomer of 2-amino-5-hydroxy-benzoyl-CoA, i.e. *5H*-2-imino-5-hydroxy-benzoyl-CoA (cf. Scheme 1, structure **II**), and A(OH)H$_2$CoA is 2-amino-5-oxocyclohex-1-enecarboxyl-CoA (cf. Scheme 1, structure **IV**).

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Abbreviations. ABzCoA-M/R, 2-aminobenzoyl-CoA monooxygenase/reductase; ABzCoA, 2-aminobenzoyl-CoA (anthraniloyl-CoA); ABzOHCoA, 2-amino-5-hydroxybenzoyl-CoA; ABzOHCoA*, 5*H*-2-imino-5-hydroxybenzoyl-CoA; A(OH)H$_2$CoA, 2-amino-5-oxocyclohex-1-enecarboxyl-CoA; MalNEt, *N*-ethylmaleimide.

Enzymes. 2-Aminobenzoyl-CoA monooxygenase/reductase (EC 1.14.13.40/1.3.1.–).

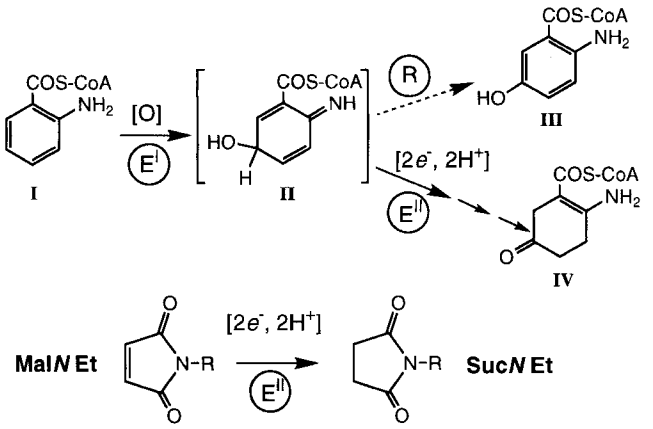
Reaction (1) is assumed to proceed according to the general mechanism common to flavoprotein hydroxylases (Ghisla and Massey, 1989), a class of enzymes which has been extensively studied in the last two decades. In contrast to this, reaction (2) is a hydrogenation of a novel type, similar perhaps to that encountered with acyl-CoA dehydrogenases (Ghisla and Massey, 1989). A primary goal of the present work was therefore a detailed study of these two reactions, and in particular to find out whether they are each associated with a single one of the two subunits of the dimeric enzyme, in contrast to a hypothetical case in which the subunits have identical active centers which can perform both reactions.

A peculiarity of ABzCoA-M/R is its capacity to use MalNEt as an electron acceptor, a reaction which might parallel the hydrogenation activity of the enzyme (Buder and Fuchs, 1989), and which is of practical use in that it provides the basis for a convenient assay. There is a structural and chemical similarity between MalNEt and the first intermediate (Scheme 1, structure **II**), which has been proposed to be formed upon oxygen insertion in ABzCoA (Langkau et al., 1990).

The approach adopted for tackling the questions outlined above involved the following experiments.

a) Exploration of the specificity of the enzyme and finding of effectors which do not participate in the monooxygenation reaction but might be useful for mechanistic studies.

Scheme 1. Reactions catalyzed by the different forms of ABzCoA-M/R. Fully active enzyme, containing both active sites E^I and E^{II} (native ABzCoA-M/R) catalyzes first mono oxygenation of ABzCoA (**I**) to yield the proposed ABzOHCoA* intermediate (**II**) at site E^I and subsequently hydrogenation at site E^{II} to yield, after two tautomerization steps, the product A(OH)H_2CoA (**IV**). In the absence of site E^{II}, such as in the case of 50% FAD-depleted ABzCoA-M/R, hydrogenation does not occur and instead rearomatization to ABzOHCoA (**III**) takes place as indicated (R, dashed arrow). The lower part of the Scheme shows the structural analogy of species **II** and *N*-ethylmaleimide (MalNEt) and the reduction of the latter to *N*-ethylsuccinimide (SucNEt) at site E^{II}. See text for further details concerning the nature of the different species.



b) Study of the NADH oxidase activity, i.e. the non-productive consumption of NADH with oxygen in the absence and presence of effectors and comparison with other hydroxylases (Powlowski et al., 1987; Spector and Massey, 1972; White-Stevens and Kamin, 1970; Yamamoto et al., 1965).

c) Photoreduction and controlled reduction of the enzyme with NADH, in the presence/absence of substrate and characterization of (half-)reduced enzyme species.

d) Reaction ('reoxidation') of half- or fully reduced enzyme with oxygen and with MalNEt.

e) Turnover and reoxidation experiments with enzyme from which FAD has been removed partially.

f) Study of the turnover with MalNEt.

A catalytic model of the NADH oxidase activity and of the turnover with MalNEt is proposed which is compatible with the experimental results. This model provides the basis for the study of the kinetic mechanism of ABzCoA-M/R with its natural substrate ABzCoA, which will be detailed in the following paper in this journal (Langkau and Ghisla, 1995).

MATERIALS AND METHODS

Chemicals and biochemicals. Biochemicals were purchased from Boehringer (Mannheim). Benzoic acid derivatives were obtained from Sigma Chemical Co. or from Fluka. *N*-Hydroxysuccinimide was from Aldrich-Chemie. All other chemicals were obtained from Riedel-de-Häen or Merck. Argon 5.0 was purchased from SWF (Friedrichshafen) and passed through an Oxisorb catalyst from Messer Griesheim (Düsseldorf) in order to remove traces of oxygen; it was humidified prior to use. 5-Bromoanthranilic acid was a gift from Dr Martin Dunkel (University of Konstanz). 4-Fluorobenzoyl-CoA, 4-aminobenzoyl-CoA and 4-nitrophenylacetyl-CoA were obtained from Dr Stefan Engst (University of Konstanz). Protocatechuate dioxygenase was a gift from Dr D. P. Ballou (University of Michigan).

Growth of organisms. The *Pseudomonas* sp. KB740$^-$ strain was grown aerobically at 28 °C in a mineral medium using anthranilate as sole carbon and energy source (Brown and Gibson, 1984). The amount of inducible ABzCoA-M/R was found to be strongly dependent on the conditions of growth and harvest of the cells. Although a detailed screening of all factors was not done, some specific factors have turned out to be important for obtaining a good yield of enzyme. A good supply of oxygen is mandatory, since under poor O_2 supply the bacteria most probably switch to an anaerobic pathway, not involving ABzCoA-M/R (Lochmeyer et al., 1992). Furthermore, the enzyme seems to be degraded very quickly when the growing bacteria enter the stationary phase. For this reason, consumption of substrate was monitored spectroscopically at 310 nm in the growth medium and 5 mM anthranilate was added one, two or three times at appropriate times to keep the concentration between 1 – 10 mM. Harvesting was carried out as quickly as possible under cooling.

Enzyme purification. 2-Aminobenzoyl-CoA monooxygenase was purified essentially according to the procedure described previously (Buder and Fuchs, 1989), except for the addition of 10 μM phenylmethylsulfonyl and 1 μM FAD at all stages before application to the DEAE-Biogel A column.

Enzyme assays. Two standard assays were used to determine ABzCoA-M/R activity. In both cases the conversion of substrate(s) was followed spectrophotometrically at 37 °C. The first is that described by Buder and Fuchs (1989). However, attention should be drawn to the observation that with fully (re)-constituted holo-ABzCoA-M/R formation of the non-reduced, aromatic product ABzOHCoA with a λ_{max} at 379 nm (Ziegler et al., 1987) could not be observed when the enzyme concentration was high enough to yield essentially complete ABzCoA conversion within ≈3 min. Thus, a different molar absorption coefficient of $\varepsilon_{365} = 11\,800\ M^{-1}\ cm^{-1}$ was used, based on the consumption of 2 mol NADH ($\varepsilon_{365} = 3400\ M^{-1}\ cm^{-1}$)/mol and 1 mol ABzCoA ($\varepsilon_{365} = 5500\ M^{-1}\ cm^{-1}$)/mol (Buder et al., 1989; Langkau et al., 1990). [NADH] was chosen as 200 μM, a concentration which was found to be saturating.

Using the second, MalNEt assay, consideration has to be given to the fact that MalNEt is an electron acceptor as well as an inhibitor of ABzCoA-M/R. This causes initial rates to become smaller with time and this, in turn, causes this assay to be less accurate than the ABzCoA assay. Hence the MalNEt assay was used preferentially to monitor enzyme activities during the purification procedure. The reaction mixture contained 100 μM NADH and 1 mM MalNEt in 100 mM potassium phosphate pH 7.8 and was started by addition of cell-free extract or of purified enzyme. The reaction was monitored by the consumption of NADH at 340 nm ($\varepsilon_{340} = 6220\ M^{-1}\ cm^{-1}$) or, for the steady-state analysis, fluorimetrically as decrease of NADH fluorescence. One unit of activity is defined as the amount of enzyme required to catalyze the oxidation of 1 μmol NADH · min^{-1} under the conditions specified.

Enzymatic synthesis. 2-Aminobenzoyl-CoA (anthraniloyl-CoA) and 2-amino[5-^{2}H]benzoyl-CoA were synthesized enzymically using a CoA ligase, which is induced in the same organism as ABzCoA-M/R (Ziegler et al., 1987). For this synthesis $(CoAS)_2$ in combination with a 10-fold excess of dithiothreitol was used instead of CoASH. The product was purified as described for the substrate analogue CoA esters (see below).

Chemical synthesis. *[5-^{2}H]Anthranilic acid.* Anthranilic acid was deuterated site-specifically by reductive dehalogenation of 5-bromoanthranilic acid with sodium borodeuteride and palladium on charcoal analogously to the method of Egli (1968). A deuterium substitution ≥85% was estimated from integration of aromatic signals in the ^{1}H-NMR spectrum.

Benzoyl-CoA (derivatives). Synthesis of substrate analogue CoA esters was achieved by transesterification of the corresponding *N*-hydroxysuccinimide esters as described by Stöckigt

and Zenk (1975). 4-Methyl-, 2-methoxy-, 4-methoxy- and unsubstituted benzoyl-*N*-hydroxysuccinimide were synthesized starting from the freshly prepared acid chlorides. However, the original reaction times (24 h) were reduced to less than 2 h. Product formation and the products were analyzed by TLC, HPLC and ultraviolet/visible spectroscopy. Starting from these activated esters benzoyl-CoA and its derivatives were synthesized by the following general procedure. The *N*-hydroxysuccinimide ester (200 μmol) was dissolved in 5 ml tetrahydrofuran and added to an aqueous solution of CoASH (50 μmol) and $NaHCO_3$ (500 μmol), which was stirred at 25 °C and flushed with nitrogen. The reaction was followed by analytical HPLC. When CoASH was consumed completely (generally within 2–3 h) the reaction mixture was acidified with 1 M HCl to pH≈3 and extracted with diethylether (10 ml). The aqueous phase was neutralized (pH 6) with concentrated LiOH and lyophilized. The CoA thioester was isolated by preparative HPLC.

Determination of absorption coefficients. Routinely, the molar absorption coefficients of newly synthesized CoA thioesters were estimated by the method of Ellman (1959). A solution of the CoA thioester (900 μl) in 20 mM potassium phosphate pH 8.0 and 1 M NaOH (100 μl) was saturated with argon prior to, and sealed immediately after, mixing in order to avoid any oxidation of the CoASH formed. Esterolysis was followed by recording ultraviolet spectra with time. The observed rates in 0.1 M NaOH varied between 0.14 min^{-1} and 0.009 min^{-1} for 4-CH_3-BzCoA and for ABzOHCoA, respectively. After completion of hydrolysis the reaction mixture was adjusted to pH 8.0 with 0.1 M HCl (100 μl) and 0.01 M 5,5′-dithobis(2-nitrobenzoic acid) (20 μl) was added. The absorption coefficient of the original CoA thioester was calculated from the *p*-nitrothiophenol anion absorbance at 412 nm (ε_{412} = 13 600 M^{-1} cm^{-1}), which resulted from the reaction with CoASH.

Chromatography. FPLC separation of highly purified native enzyme was performed on a Pharmacia system with LKB 2150 HPLC pump, LKB 2152 HPLC controller and LKB 2210 variable wavelength monitor. Protein elution was monitored at 280 nm. For analytical and preparative purposes a Mono Q 5/5 (flow rate 1.5 ml/min) or a Mono Q 10/10 column (5 ml/min) was used. They were equilibrated with 50 mM Tris/HCl pH 7.5 containing 0.02 mM EDTA and 0.02 mM dithioerythritol (buffer A). Buffer B contained 1 M NaCl in buffer A. Five fractions were isolated by chromatography on preparative Mono Q and marked as indicated in Fig. 3. The formation of species 2 from fractions 1 and 3 was studied by mixing and storing the latter at 4 °C in 50 mM Tris/HCl pH 7.5 containing 0.02 mM EDTA and 0.02 mM dithioerythritol. Aliquots were analyzed with time by chromatography on Mono Q 5/5.

CoA-thioesters were routinely analyzed by HPLC using an RP-18 LiChrospher column (250 mm×4 mm; Merck) and a gradient of 10–45% (by vol.) aqueous methanol (95%) in 20 mM sodium phosphate pH 6.0. The CoA-thioesters were purified by preparative HPLC using an RP-18 Spherisorb column (250 mm×20 mm; Bischoff, Leonberg, FRG) and a gradient of 20–55% (by vol.) in the same solvent system.

Analysis of products. Solutions of NADH (200 μM) and ABzCoA (100 μM) were reacted under standard assay conditions (Buder and Fuchs, 1989) with purified ABzCoA-M/R and each of the three main enzyme fractions obtained from Mono Q. For analysis of the product distribution between ABzOHCoA and A(OH)H_2CoA, the enzyme was separated by Centricon ultrafilters (Amicon) and the reaction mixture was purified by HPLC as described for the CoA esters.

Binding of FAD. The K_d for FAD binding was studied using the method of Dixon and Kleppe (1965) by assaying ABzCoA-M/R activity at 37 °C as function of time upon preparing dilutions of enzyme at concentrations in the range 1–0.01 μM. The K_d was determined by comparing the activity measured at the end of changes with the initial activity. The velocity of FAD dissociation was estimated from the initial rate of decrease of activity.

Reduction/reoxidation experiments. All reduction and reoxidation experiments were performed in 50 mM sodium or potassium phosphate pH 7.4 at 4 °C. For anaerobiosis 0.8–1 ml freshly filtered (0.45-μm filters from Millipore Corp.) enzyme solution was placed in the main compartment of an anaerobic cuvette. Small volumes, typically 20 μl of the reductant and/or oxidant, were present in the sidearm(s). The cell was made anaerobic at 0 °C by up to eight cycles of evacuation and flushing with purified argon. The cell was kept under vacuum during the experiments unless oxygen was admitted at the times indicated. Anaerobic reactions were started by mixing the reactant, i.e. NADH or MalNEt from one sidearm. Spectral changes were followed spectrophotometrically and those which occurred within the mixing time are represented by dashed lines in Figs 6, 7, and 8. EDTA/*hv* reductions were performed at 4 °C using a 250-W halogen lamp at a distance of ≈15 cm and irradiation periods ≤1 min using the set-up described earlier (Massey et al., 1978).

Instrumentation. Absorption spectra and single-wavelength kinetics were recorded either with a Kontron-Uvikon 920 spectrophotometer or with a Hewlett-Packard 8452A diode array detector. HPLC and ^{1}H-NMR measurements were made as described earlier (Langkau et al., 1990). Fluorescence was measured with a Kontron SFM-25 fluorimeter.

RESULTS

Stability. While the enzyme is stable when stored in liquid nitrogen, when kept as a micomolar solution, at pH 7.8 and at 0 °C or 25 °C it gradually looses activity with an apparent $t_{1/2}$≈2 days. No apparent stabilization is achieved in the presence of substrate ABzCoA, NADH or FAD. In dilute solution (≈10 nM) and at 25 °C the loss of activity has a $t_{1/2}$≈2 min, which is substantially enhanced ($t_{1/2}$≈1–1.3 min) in the presence of 2 mM MalNEt. At this dilution the presence of ≈100 μM concentrations of either NADH, ABzCoA or FAD enhances stability considerably ($t_{1/2}$≈30 min). These results suggest that inactivation is connected with loss of FAD cofactor (cf. also below) and that MalNEt is an inactivator probably reacting preferentially with the apo-protein.

Substrate and effector specificity. The catalytic velocity of NADH consumption in the presence of ABzCoA (Scheme 1, structure **I**) and a number of analogous CoA thioesters was studied by initial rate measurements under aerobic conditions (Table 1). Highest rates were found with ABzCoA and [5-^{2}H]ABzCoA, corresponding to an ≈150-fold enhancement compared to the oxidase activity of unliganded enzyme. A significant acceleration was also observed with the 2-hydroxy analog of ABzCoA (2-OH-BzCoA) (40-fold) and ABzOHCoA (20-fold), while BzCoA and the 4-fluoro analog (4-F-BzCoA) showed only relatively small effects. With other analogs, 4-CH_3-BzCoA, 3-NH_2-BzCoA, 4-NH_2-BzCoA, 4-OH-BzCoA, 2-OCH_3-BzCoA and 4-NO_2-phenylacetyl-CoA, the velocity of NADH oxidation was enhanced less than 2-fold. In order to distinguish between substrates and non-substrate effectors the extent of conversion was analyzed by HPLC. Only ABzCoA and [5-^{2}H]ABzCoA were converted completely, yielding 2-amino-5-oxocyclohex-1-enecarboxyl-CoA as the main product (Langkau et al., 1990). 2-OH-BzCoA is converted to ≈10% into a product

Table 1. Consumption of NADH and production of H_2O_2 by ACoA-M/R in the presence of substrate and non-substrate effectors. V_{max} is defined as the consumption of NADH in incubations containing 100 μM substrate/effector, 200 μM NADH and 60 nM E~(FAD)$_2$ in 100 mM potassium phosphate pH 7.8 at 25°C. The decrease in absorbance was followed at 365 nm using $\Delta\varepsilon_{365}$ = 3.18 mM^{-1} cm^{-1} for the consumption of NADH with non-substrate effectors or $\Delta\varepsilon_{365}$ = 5.93 mM^{-1} cm^{-1} for the conversion of 1 mol NADH/0.5 mol ABzCoA or [5-^{2}H]ABzCoA, respectively. One unit (U) is defined as mol NADH oxizied · min^{-1} · mol enzyme dimer^{-1}. Samples were withdrawn before and after the reaction and analyzed by HPLC for consumption of the thest compound. The production of H_2O_2 due to the effectors is the difference in oxygen detection between a reaction performed in the presence of catalase and one in the absence. In the second case catalase was also added upon completion of the ABzCoA-M/R-catalyzed reaction and the amount of oxygen produced secondarily correlated to that of H_2O_2. Conditions: cells contained 100 μM NADH, 130 μM effector and ≈1400 U catalase in 100 mM potassium phosphate pH 7.8 in a final volume of 2.1–2.2 ml. Reactions were initiated by addition of 0.12–0.24 nmol ABzCoA-M/R; n.d. = not determined.

Substrate/effector	V_{max}	Mono-oxygenation	Formation of H_2O_2
	U		%
None	5		n.d.
ABzCoA	824	+++	0
[5-^{2}H]ABzCoA	817	+++	10 ± 4
2-OH-BzCoA	214	+	92 ± 2
ABz(OH)CoA	112	+/−	91 ± 7
BzCoA	33	−	94 ± 6
4-F-BzCoA	29	−	n.d.

which has a shorter retention time on RP-18 HPLC indicating a higher polarity, and which might be a dihydroxybenzoyl-CoA ester. The partial conversion of ABzOHCoA is attributed to spontaneous hydrolysis of the CoA ester rather than to hydroxylation. A possible hydrogenation of ABzOHCoA (Scheme 1, **III**) to form e.g. 2-amino-5-oxocyclohex-1-enecarboxyl CoA (Scheme 1, **IV**) is very unlikely, as judged from absorption spectra of the product mixture. BzCoA and 4-F-BzCoA remained entirely unaffected during NADH oxidation. Some substrate analogs are known to promote NADH consumption to form H_2O_2 without monooxygenation (uncoupling, White-Stevens and Kamin, 1970). The uncoupling of NADH consumption from hydroxylation in the cases listed in Table 1 was verified by the procedure of White-Stevens and Kamin (1970). In agreement with product analysis by HPLC BzCoA, 2-OH-BzCoA and ABzOHCoA yielded an almost 50% reformation of O_2 from H_2O_2 upon addition of catalase (Table 1), which is consistent with a predominantly 'non-substrate' or 'effector' activity. With ABzCoA no formation of H_2O_2 could be detected while with its deuterated form [5-^{2}H]ABzCoA traces of H_2O_2 were found.

Binding of the substrate ABzCoA and of analogs to oxidized and reduced ABzCoA-M/R. The addition of ABzCoA to oxidized enzyme causes large and marked perturbations of the visible absorption spectrum of the flavin chromophore (Fig. 1A). The spectral changes observed consist of two phases. The first one is essentially complete experimentally at a ≈1.2-fold excess of ABzCoA compared to enzyme-bound flavin, while the second is not saturated at a 12-fold excess and shows an apparent K_d≈700 μM. Since the K_m for ABzCoA has been reported to be < 25 μM and substrate inhibition occurs at [ABzCoA] > 150 μM (Buder et al., 1989), it is likely that the weak binding has no catalytic relevance, but is associated with inhibition. Titration

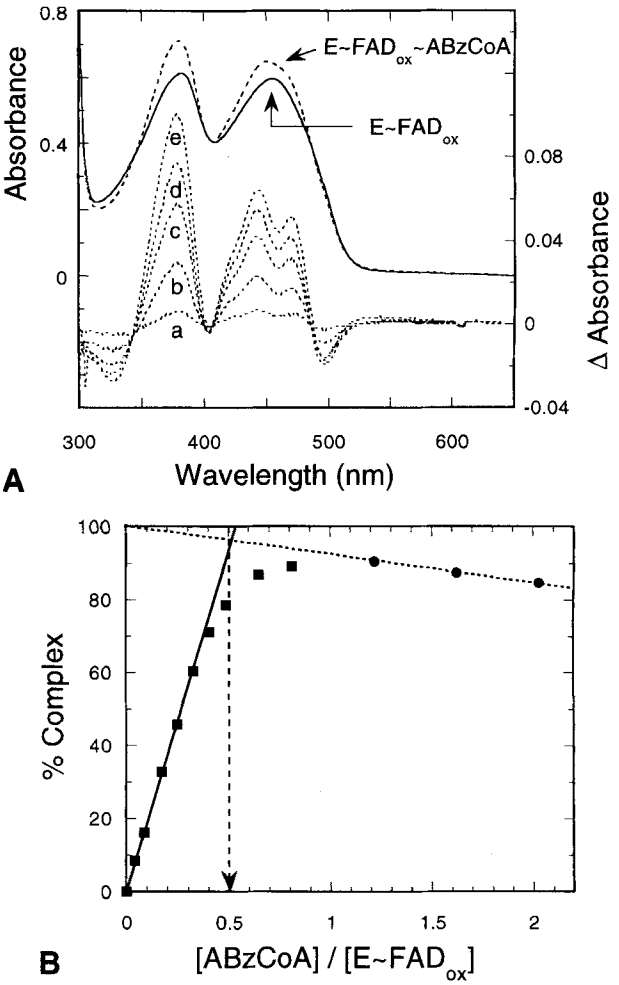


Fig. 1. Binding of ABzCoA to oxidized ABzCoA-M/R (E~FAD$_{ox}$). (A) Spectral perturbations accompanying binding. Conditions: 53 μM enzyme-bound flavin (E~FAD), 0.1 mM EDTA in 1.0 ml 50 mM sodium phosphate pH 7.4 at 4°C. All spectra were recorded versus an equal concentration of substrate in the reference cuvette and are corrected for dilution. The scale on the left ordinate corresponds to the spectra of oxidized ABzCoA-M/R before (——) and upon addition of 63 μM ABzCoA (1.2-fold molar excess) (– – –). Calculated difference spectra a–e (·····) were obtained upon additions of 2.3 μM, 8.9 μM, 17 μM, 25 μM, and 63 μM ABzCoA (right ordinate scale). Peak maxima of positive absorbance changes were at 471, 443 and 378 nm, and of negative changes at 497 and 327 nm. Isosbestic points were at 487, 408, 401 and 346 nm. Addition of ≈600 μM ABzCoA resulted in a second set of difference spectra characterized by decreasing absorbance changes at all maxima shown, except for that at 378 nm (not shown). See text for further details. (B) Stoichiometry of binding. Data obtained from similar experiments are plotted as the percentage of complete complex formation (E~FAD$_{ox}$~ABzCoA) versus the ratio [ABzCoA]/[E~FAD$_{ox}$]. 100% E~FAD$_{ox}$~ABzCoA for the first binding site was estimated from back-extrapolation of the absorbance changes at 443 nm occurring in the second phase (– – –) which reflects binding of further ABzCoA to a second site (●). Spectral perturbations resulting from substoichiometric additions of ABzCoA and binding to the first site are also shown (■).

with ABzCoA indicates a stoichiometry of only 0.5 mol ABzCoA/mol enzyme flavin for the primary binding and a K_d≤1 μM (Fig. 1B). Additions of ABzCoA to E~FADH$_2$ also caused spectral perturbations compatible with binding to a half-site (Fig. 2) and a K_d≤2 μM. It is most important to note that fully reduced ABzCoA-M/R does not react with (hydrogenate) ABzCoA.

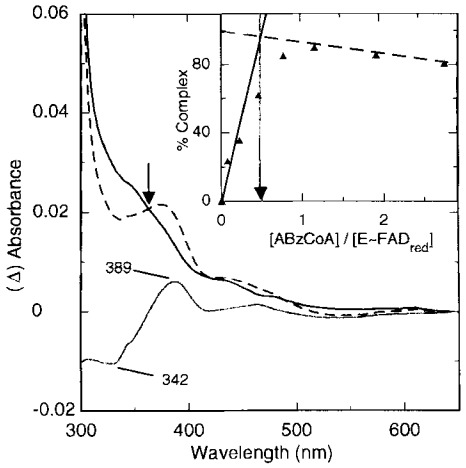


Fig. 2. Binding of ABzCoA to reduced ABzCoA-M/R (E ~ FAD$_{red}$). (——) Absorbance of uncomplexed E ~ FAD$_{red}$; (– – –) absorbance of E ~ FAD$_{red}$ ~ ABzCoA complex obtained upon addition of 1.1 mol ABzCoA/mol complex; lower curve, the difference spectrum between the preceding two. The arrow indicates an isosbestic point at 355 nm. The insert shows the determination of the stoichiometry of ABzCoA binding done as outlined in the legend of Fig. 1. The absorbance changes between 389 nm and 342 nm were used for the estimation of 100% E ~ FAD$_{red}$ ~ ABzCoA complex. Conditions: 17.3 μM E ~ FAD (enzyme-bound flavin), containing 10 mM EDTA, 0.5 μM 5-deazariboflavin, 100 μM protocatechuate and 0.46 mU protocatechuate dioxygenase in 50 mM potassium phosphate pH 7.4 under anaerobic conditions. The solution was stored overnight in order to consume residual oxygen in the system and and was then illuminated until the spectral changes accompanying reduction were complete. ABzCoA in an argon-saturated solution was then added with a syringe through a septum in seven portions yielding a final [ABzCoA] = 47.5 μM. The presence of traces of oxygen required occasional irradiation upon addition of ABzCoA in order to restore full reduction. This might have caused turnover of up to 20% of the total [ABzCoA] and thus a corresponding error in the K_d determination. The estimated apparent $K_d \leq 1.8$ μM is therefore an upper limit.

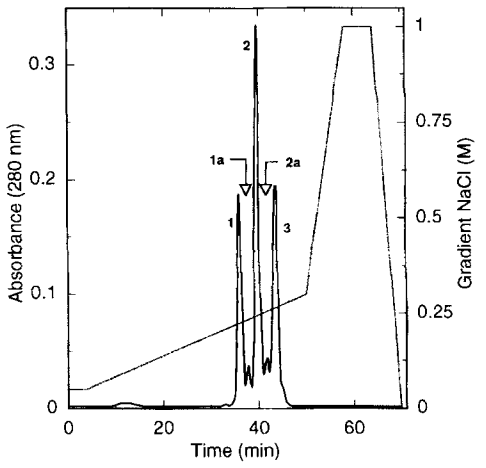


Fig. 3. Fast protein liquid chromatography on Pharmacia Mono Q of purified ABzCoA-M/R. 5 mg highly purified native enzyme obtained as described in Materials and Methods was chromatographed on a Mono Q 10/10 column. Starting buffer A: 50 mM Tris/HCl pH 7.5 containing 0.02 mM EDTA and 0.02 mM dithioerythritol; buffer B: buffer A plus 1 M NaCl. Flow rate 5 ml/min. Gradient as shown by the dashed line. Three major (1, 2 and 3) and two minor (1a, 2a) protein peaks were collected separately.

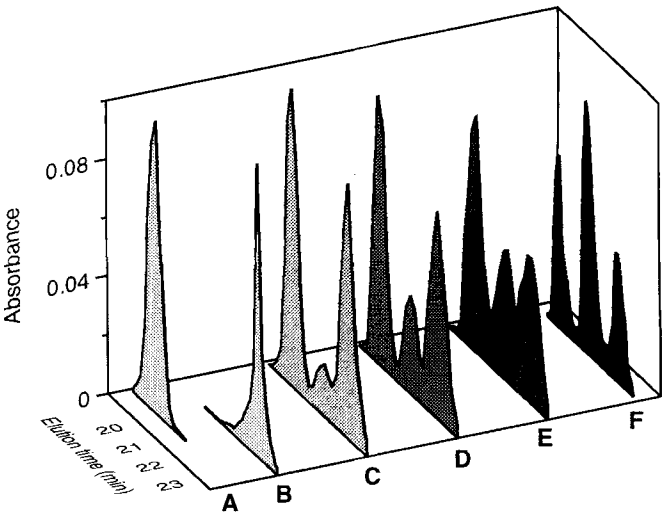


Fig. 4. Recombination of ABzCoA-M/R species 1 and 3 to form species 2. The purity of isolated species 1 and 3, obtained as described in Fig. 3 (see also text for details), is shown by chromatograms A and B. These species were mixed in approximately equal amounts in buffer A at 4 °C and the incubation analyzed immediately upon mixing (t = 0 h, profile C), and at t = 45 h (D) and 96 h (E). At longer incubation times some protein denaturation occurs. The profile of native untreated enzyme is shown for comparison (F).

The substrate analogs BzCoA, 2-OH-BzCoA and the product ABzOHCoA also bind to oxidized ABzCoA-M/R and perturb its absorption spectrum. 0.5 mol BzCoA binds to 1 mol ABzCoA-M/R dimer with a $K_d \approx 19$ μM and to the second site with $K_d \approx 330$ μM. With ABzOHCoA the first binding step has the same stoichiometry and a $K_d \approx 1$ μM, the second was substantially larger, but could not be determined due to the low quantity of material available. With 2-OH-BzCoA the spectral perturbations observed are also compatible with a biphasic binding and with a stoichiometry of 0.5 mol/mol ABzCoA-M/R dimer, however they do not allow a determination of the second step probably due to a very large K_d.

Resolution of purified ABzCoA-M/R by chromatography on Pharmacia Mono Q anion exchanger and FAD binding. Fig. 3 shows a typical profile observed when pure ABzCoA-M/R was eluted from a preparative Mono Q anionic-exchange column. Three major fractions designated 1, 2 and 3, with a relative absorbance ratio of approx. 1:2:1 were observed, which correspond to what has been described previously by Buder and Fuchs (1989). However, two further minor species (fractions 1a, 2a) were also observed. All five fractions exhibited ABzCoA-M/R activity. Upon mixing fractions 1 and 3, the conversion of part of the protein to species 2 occurs as shown in Fig. 4. This is in agreement with the finding of Buder and Fuchs (1989) that rechromatography of species 2 yields an elution profile qualitatively as shown in Fig. 3, i.e. that species 2 can yield species 1 and 3. Treatment of ABzCoA-M/R with either a 10-fold excess of H_2O_2 for 12 h or a 100-fold excess of dithioerythritol for 24 h, both at 4 °C, did not modify the elution profile. The H_2O_2 treatment, however, essentially abolished activity and both the absorption and fluorescence spectra of FAD were affected. Six SH-groups (three pairs) react with 3 mol 5,5′-dithiobis(2-nitrobenzoic acid)/mol ABzCoA-M/R monomer, with complete loss of activity and of FAD binding, as determined by ultrafiltration.

The three main species contained in peaks 1, 2 and 3 differ markedly in their specific activities and in their ratios of absorption $A_{280/450}$, which reflects the proportion of holo- to apo-protein (Table 2). For the best fractions of ABzCoA-M/R obtained from

Table 2. Specific activity of ABzCoA-M/R species 1, 2 and 3 and comparison with FAD content. The FAD content was measured as the $A_{280/450\,nm}$ ratio). Species are as defined in Fig. 3, specific activity as defined in Table 1. Reconstitution was performed by incubation at 25°C for 10 min with a 100-fold molar excess of FAD to obtain full FAD content.

ABzCoA-M/R species	Isolated enzyme				Reconstituted enzyme			
	FAD	specific activity with			FAD	specific activity with		
		ABzCoA	MalNEt	MalNEt/ ABzCoA		ABzCoA	MalNEt	MalNEt/ ABzCoA
	$A_{280/450}$	U		ratio	$A_{280/450}$	U		ratio
Untreated	9.2	1780	5820	3.3	9.2	1780	5820	3.3
1	11.8	1760	5300	3.0	9.6	1780	5840	3.3
2	12.8	1720	4970	2.9	10.2	1760	5670	3.2
3	17.6	620	600	1	10.9	1760	5500	3.1

hydroxyapatite, which were not chromatographed on Mono Q, the $A_{280/450}$ was typically 9.2. This ratio increases from species 1 to 3 suggesting a decrease of FAD content. Thus fraction 3 can be estimated to contain 50–55% FAD ($A_{280/450}$ = 17.6) as compared to ABzCoA-M/R before Mono-Q chromatography. Surprisingly, the specific activities of the three ABzCoA-M/R fractions, calculated on the basis of the FAD content, differ from those of unresolved enzyme when measured either with MalNEt or with ABzCoA as substrate: Fraction 3 has only ≈10% of the MalNEt and ≈30% of the ABzCoA activity compared to unresolved enzyme. Hence, the ratio of the specific activities found for MalNEt/ABzCoA dropped from 3.3 for untreated enzyme to 1.0 for fraction 3, fractions 1 and 2 being intermediate cases (cf. Table 2). This is in agreement with the finding of Buder and Fuchs (1989) that rechromatography of species 2 yields an elution profile qualitatively as shown in Fig. 3, i.e. that species 2 can yield species 1 and 3.

Most importantly there is a difference in the product distribution when ABzCoA is turned over by untreated ABzCoA-M/R compared to fractions 1, 2 or 3 (Table 2). Products were separated from NAD^+/NADH and ABzCoA-M/R and analyzed as shown in Fig. 5. Turnover of ABzCoA (**I**) using unresolved ABzCoA-M/R appears to yield essentially the normal product $A(OH)H_2CoA$ (**IV**) while with fraction 3 the relative content of ABzOHCoA (**III**) is ≈60%. At first glance these results suggest that species 1, 2, and 3 are competent in monooxygenation, but vary in their capacity to hydrogenate. The relative differences between these activity profiles can readily be explained by loss of FAD selectively at the active site catalyzing hydrogenation. Thus ABzCoA-M/R completely depleted of FAD at its 'hydrogenation site' should catalyze only formation of ABzOHCoA (**III**). In agreement with this we find mainly ABzOHCoA (**III**) formation (up to 60%) using fraction 3. FAD 'loss' by species 1,2 and 3 could be reversed by incubation with a 100-fold excess of FAD for 1 h when activity comparable to that of native enzyme was recovered (Table 2). Using the method of Dixon and Kleppe (1965), the dissociation rate (k_{off}) for FAD to yield 50% FAD-depleted enzyme was estimated as 0.7–1.5 min^{-1} which corresponds to the rate of inactivation of dilute enzyme solutions. The corresponding K_d for FAD dissociation was ≈0.06 μM at 37°C assuming that ≈0.5 mol FAD dissociate/mol enzyme dimer (same method, data not shown). In fact monooxygenase activity was not decreased below ≈30% of the initial value even at the lowest enzyme dilutions, in contrast to the hydrogenase activity.

Reduction and (re)oxidation experiments. *Photoreduction.* Light irradiation of ABzCoA-M/R in the presence of EDTA yields fully reduced enzyme without formation of detectable amounts of the red or blue semiquinone species as intermediates (not shown). On the basis of ε_{454} = 11.3 $mM^{-1} \cdot cm^{-1}$ for oxidized enzyme (Buder and Fuchs, 1989), the absorption coefficient for reduced enzyme was estimated as ε_{454} = 1.84 $mM^{-1} \cdot cm^{-1}$. The presence of 1 μM 5-deazariboflavin enhances the rate of photoreduction as found also for other flavin enzymes (Massey and Hemmerich, 1977). Reoxidation occurs upon admission of oxygen; the kinetic course of this process will be described below.

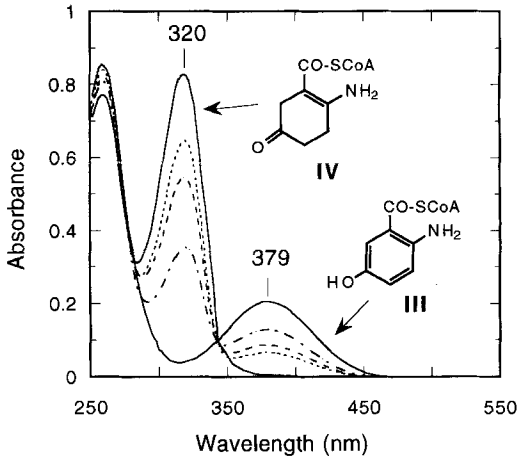


Fig. 5. Ultraviolet/visible absorption spectra of product mixtures resulting from incubations of ABzCoA with untreated ABzCoA-M/R, and with species 1 or 3. Product mixtures were separated from excess substrate and NAD^+/NADH by HPLC and analyzed subsequently by spectroscopy. Curves were obtained from incubations using (·····) native enzyme, (– – –), species 1 and (– · – ·) species 3 indicating formation of 71%, 62% and 39% $A(OH)H_2CoA$, respectively; (——) spectra of pure $A(OH)H_2CoA$ (λ_{max} = 320 nm) and of ABzOHCoA (λ_{max} = 379 nm) shown for comparison.

Reduction with NADH. When ABzCoA-M/R is reacted anaerobically with a 13-fold excess of NADH in the absence of the substrate ABzCoA the ensuing reaction is markedly biphasic, about half the oxidized flavin absorption disappearing within the mixing time of the reactants. The reduction of the remaining half occurs at a much slower rate of ≈0.06 min^{-1} as shown in Fig. 6, curve A. In contrast, the presence of stoichiometric [ABzCoA] results in ≈85% reduction within the mixing time, while the residual part is reduced at about the same rate as the slow phase in the absence of ABzCoA (Fig. 6, curve B, ▲). The observation of ≈15% slow reaction is interpreted as being due

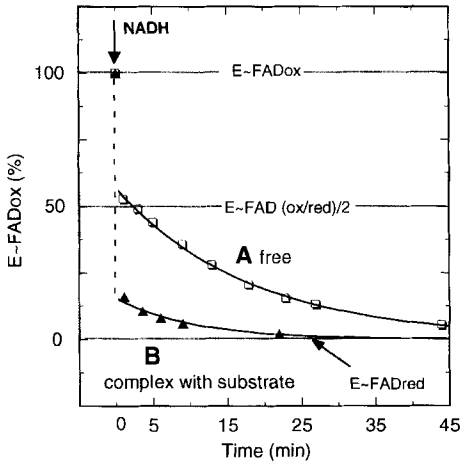


Fig. 6. Course of NADH reduction of ABzCoA-M/R in the absence and presence of ABzCoA. In experiment (A) 200 μM NADH (final concentration) was added from one sidearm of an anaerobic cuvette to 15.4 μM enzyme (E ~ FAD) at 4 °C and the reduction was monitored starting within 0.5 min of mixing the reactants. Almost 50% of the total absorbance of oxidized flavin measured at 454 nm disappeared within this mixing time (- - -); (□) the subsequent decrease; (——) the fit of the data points assuming a pseudo-first-order reaction with $k_{obs} = 0.06$ min^{-1}. The companion experiment (B, ▲) was performed analogously using 10.8 μM enzyme (E ~ FAD) which, however, was complexed with 11.3 μM ABzCoA at 4 °C. The reduction was started by adding 192 μM NADH (final concentration) from the sidearm of the anaerobic cuvette. Bleaching of E ~ FAD_{ox} ~ ABzCoA at 454 nm was about 85% complete within the mixing time. The residual ≈15% of oxidized enzyme was reduced at approximately the same rate as in the slow phase in the absence of substrate.

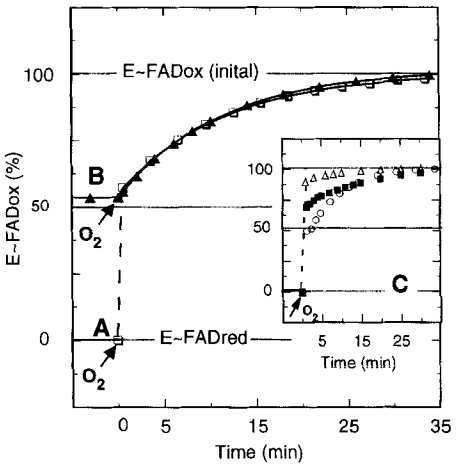


Fig. 7. Oxygen (re)oxidation of fully and half-reduced ABzCoA-M/R. (A) 8.9 μM enzyme (E ~ FAD) was made anaerobic and reduced photochemically at 4 °C in the presence of 12 mM EDTA. Reoxidation was initiated by admission of air to the anaerobic solution and spectral changes were followed at 454 nm. Some 55% of the reduced enzyme, as deduced from the absorbance increase at 454 nm, was reoxidized within the mixing time of < 0.5 min, and the remaining with a $k_{obs} \approx 0.1$ min^{-1}. (——) The fit of the data points (□) assuming a pseudo-first-order reaction. (B) Similar experiment in which enzyme was reduced to approximately 50% by titration with NADH before admission of O_2. The observed rate of reoxidation is approximately the same as that of the slow phase in A. The insert (C) shows the traces obtained from similar experiments obtained using ≈ 20 μM native enzyme (○), species 2 (■) and 3 (△) treated as described in A. The percentage oxidized enzyme flavin obtained within the mixing time was 50%, 70% and 90% for native enzyme, species 2 and species 3, respectively.

to the presence of the corresponding amount of uncomplexed ABzCoA-M/R. Together with the observation that one enzyme dimer binds tightly one ABzCoA molecule (i.e. stoichiometry of 0.5 mol ABzCoA/mol FAD), these results suggest that substrate binds to, and affects the NADH reactivity of, one specific 'half' of the enzyme flavin. In the following paper (Langkau and Ghisla, 1995) direct evidence will be presented that indeed ABzCoA binding does not affect the NADH reactivity of 'the other half' of the flavin population.

Reaction of reduced enzyme with oxygen in the absence of ABzCoA. The course of reoxidation of fully (photo)reduced, uncomplexed enzyme is also strongly biphasic (Fig. 7, A, □), with a 1:1 ratio of the fast and slow phase ($k_{obs} = 0.1$ min^{-1}). When ABzCoA-M/R was first reduced to ≈50% by addition of limited NADH and subsequently exposed to oxygen, a monophasic and slow reaction (same rate as above) was observed (Fig. 7, B, ▲). These results are compatible with the presence of two sites which have different reactivities towards oxygen and are independent of each other. Furthermore, it can be concluded that the site which is reduced rapidly by NADH (in the absence of substrate) is the one which is reoxidized slowly by oxygen and vice versa. The same type of 'reoxidation' experiments was performed using species 1, 2 and 3 (as defined in Fig. 3), which have a varying content of FAD cofactor. The results show that the percentage of enzyme flavin reacting very fast with O_2 (Fig. 7, C) increases in that order, i.e. it correlates inversely with the FAD content remaining in the hydrogenation active site. This corresponds to the ratio of ABzOHCoA/A(OH)H_2CoA formed using species 1, 2 and 3, which also increases with decreasing FAD content. As an extension of the above interpretation, it can be deduced that ABzCoA-M/R has two different active sites, one catalyzing the monooxygenation which reacts very rapidly with oxygen, and the other which catalyzes the hydrogenation and reacts slowly with oxygen.

Reaction of reduced enzyme with MalNEt in the absence of ABzCoA. When fully reduced enzyme is mixed anaerobically with MalNEt, ≈50% of the enzyme flavin, corresponding to one half-site of ABzCoA-M/R, is reoxidized quickly while the remaining 50% is reoxidized very slowly ($t_{1/2}$ on the scale of hours). There is, however, a substantial difference as compared to the reaction with oxygen: the reoxidation of NADH-half reduced enzyme by MalNEt occurs within the mixing time while that with oxygen is slow (Fig. 7, B). This supports the concept of two sites, which differ substantially in their rates of reduction and their reactivity with oxygen and MalNEt respectively. This complementarity of the two sites is supported directly by the results of the experiment shown in Fig. 8. Addition of first MalNEt and subsequently of oxygen to fully reduced enzyme leads to oxidized enzyme in two fast steps.

Reactions of E ~ FAD_{red} ~ ABzCoA with oxygen and/or MalNEt. The O_2 reaction of the fully reduced ABzCoA-M/R ~ ABzCoA complex leads to formation of product(s) as represented by Eqn (3) or Scheme 1 and involves quite a complicated sequence of steps as will be detailed in the following paper (Langkau and Ghisla, 1995). Addition of MalNEt to ABzCoA-M/R_{red} ~ ABzCoA leads to rapid (re)oxidation of ≈50% of the enzyme flavin, and subsequent addition of O_2 to rapid formation of fully oxidized ABzCoA-M/R. This is qualitatively what is observed in the absence of ABzCoA (compare Fig. 8), the difference being the formation of aromatic product (Scheme 1, **III**). The sequence is represented by Eqn (4). In contrast to this, when the substrate complex of half-reduced enzyme (E ~ $FAD_{red/2}$ ~ ABzCoA) is reacted with O_2 only slow reoxidation ensues and substrate does not react according to Eqn (5) (E ~ $FAD_{red/2}$ ~ ABzCoA was obtained by titration with NADH up to the half-

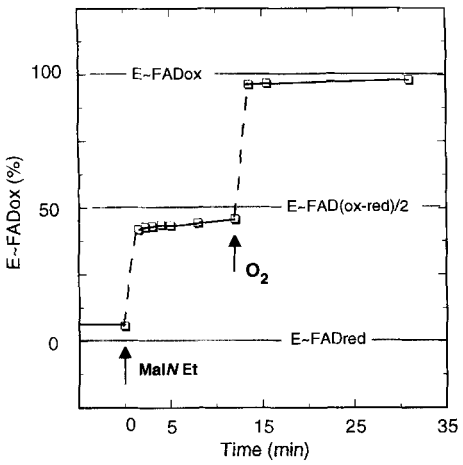


Fig. 8. Reoxidation of NADH-reduced ABzCoA-M/R by MalNEt and oxygen. Reduction conditions were as described in Fig. 6 (A) and at ≈45 min 95% reduction was achieved. 20 µl MalNEt (final concentration 2 mM) were then added from a second side arm and this caused ≈40% reoxidation within the time of mixing (≈1 min) as monitored from the absorbance increases at 454 nm, followed by minor spectral changes within the next 12 min. O_2 was then admitted yielding >95% reoxidized enzyme in less than 1.5 min.

reduced state and subsequent addition of ABzCoA). This result corresponds to that which is observed in the absence of ABzCoA as shown in Fig. 7. In these experiments formation of products **III** and **IV** (Scheme 1) can be deduced by their absorption in the near-ultraviolet (Langkau et al., 1990) and perturbation of the spectrum of oxidized ABzCoA-M/R.

These results are again consistent with the occurrence of two different flavin sites, which do not exchange redox equivalents on the time scale relevant for catalysis. Furthermore they show that in the course of formation of **IV** monooxygenation precedes hydrogenation.

$$E\sim FAD_{red}\sim ABzCoA \xrightarrow[fast]{O_2} E\sim FAD_{ox} + \text{products (III, IV)} \quad (3)$$

$$E\sim FAD_{red}\sim ABzCoA \xrightarrow[fast]{MalNEt} [E\sim FAD_{red/2}\sim ABzCoA] \xrightarrow[fast]{O_2} E\sim FAD_{ox} + ABz(OH)CoA\ (\mathbf{III}) \quad (4)$$

$$[E\sim FAD_{red/2}\sim ABzCoA] \xrightarrow[slow]{O_2} E\sim FAD_{ox} + ABzCoA\ (\mathbf{I}). \quad (5)$$

Steady-state analysis of the reaction with MalNEt. The enzyme-catalyzed oxidation of NADH with MalNEt serving as an artificial electron acceptor was investigated by initial rate measurements. Since the oxidase activity of the enzyme is considerably lower compared to turnover with MalNEt the experiments could be performed under aerobic conditions and analyzed as a classical two-substrate enzyme-catalyzed reaction. Consideration has to be given to the fact that MalNEt is also an inhibitor of ABzCoA-M/R in addition to its capacity as an electron acceptor. This causes initial rates to become slower with increasing incubation time. The steady-state equation for an enzyme-catalyzed reaction involving two substrates (NADH and MalNEt) is

$$e/v = \phi_0 + \frac{\phi_{NADH}}{[NADH]} + \frac{\phi_{MalNEt}}{[MalNEt]} + \frac{\phi_{MalNEt,\,NADH}}{[MalNEt][NADH]} \quad (6)$$

in which e is the concentration of active centers and v is the observed initial rate. A set of virtually parallel lines was obtained in a Lineweaver-Burk plot of initial velocities versus the concentration of NADH at various levels of MalNEt (data not shown). A secondary plot of the y-axis intercepts versus reciprocal MalNEt concentration also yielded a straight line indicating that the last term in Eqn (6) approaches zero ($\phi_{MalNEt,NADH} \approx 0$), and are consistent with the occurrence of a 'ping-pong' or enzyme-substitution mechanism. The kinetic constants obtained from the various plots are: V_{max} = 6550 min^{-1} ($1/\phi_0$), $K_{m(NADH)}$ = 26 µM (ϕ_{NADH}/ϕ_0) and $K_{m(MalNEt)}$ = 0.47 mM (ϕ_{MalNEt}/ϕ_0; for conditions see Materials and Methods).

DISCUSSION

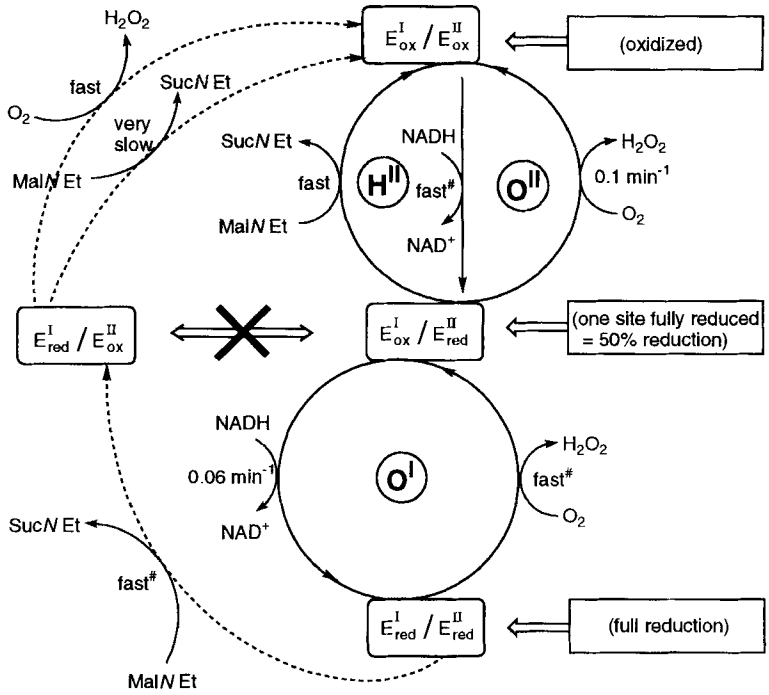
The puzzling observation of Buder and Fuchs (1989) that ABzCoA-M/R can separate into three active fractions upon chromatography on Mono Q can now be partially rationalized. Our results are compatible with the interpretation of these authors, that there must be at least two ionically different species of ABzCoA-M/R-monomers α and α' which can form the dimer species $\alpha\alpha$, $\alpha\alpha'$ and $\alpha'\alpha'$. Additionally, the finding of two minor peaks 1a and 2a (see Fig. 3) suggests the existence of further species. The molecular differences between α and α' have not yet been identified. However, it is clear that the microheterogeneity does not result from oxidation of SH-groups, as was described e.g. for *p*-hydroxybenzoate hydroxylase (Van Berkel and Müller, 1987) since incubation of ABzCoA-M/R with H_2O_2 does not result in a change of the peak ratios upon chromatography on Mono Q ion-exchange resin. Furthermore there is no correlation of the microheterogeneity with the different activities of the two active sites. The five species show closely similar activities with ABzCoA as well as with MalNEt upon reconstitution to full FAD content. The differences of species α and α' are thus caused by ionic heterogeneity of the protein and this, in turn, appears to affect directly or indirectly binding of FAD.

The different site reactivities observed with ABzCoA-M/R in the absence of substrate are consistent with the sequences of Scheme 2. This includes two active sites E^I and E^{II} which differ in their reactivities towards NADH, MalNEt and O_2. Fast reduction of oxidized enzyme with NADH in the absence of the aromatic substrate occurs at the site labelled E^{II} and yields the half-reduced enzyme species (E^I_{ox}/E^{II}_{red}). E^{II} is also the site which reacts readily with MalNEt to reduce it. These two processes result in high activities for the consumption of NADH and MalNEt under steady-state conditions and are represented by the 'hydrogenation cycle' H^{II}. The site E^{II}, however, reacts only very slowly with oxygen, resulting in a low oxidase activity as represented by cycle O^{II}.

The companion site E^I is, in contrast, only slowly reduced by NADH in the absence of ABzCoA, but it is reoxidized readily by oxygen, providing the basis for a second low oxidase activity via cycle O^I. Oxidation of E^I_{red} is negligible with MalNEt, but very fast with oxygen. In agreement with this, MalNEt reacts rapidly with the E^{II}_{red} site of fully reduced enzyme (E^I_{red}/E^{II}_{red}), to produce the half-reduced species (E^I_{red}/E^{II}_{ox}). The latter cannot be obtained directly from E^I_{ox}/E^{II}_{ox} by selective reduction of E^I_{ox}, it is thus substantially different from E^I_{ox}/E^{II}_{red}. As judged from the results of the reoxidation experiments described, there cannot be any appreciable interconversion of the species E^I_{ox}/E^{II}_{red} and E^I_{red}/E^{II}_{ox}. This suggests that a (kinetic) barrier prevents exchange of redox equivalents between the two cofactors. The latter could be located at sufficient distance from each other, or might not have a favorable reciprocal orientation.

Since O_2 reacts efficiently only at the E^I_{red} but not at the E^{II}_{red} site, the sequence represented by the dashed lines in Scheme 2 is viable for oxygen consumption only in the presence of MalNEt, which oxidizes E^{II}_{red} much faster than E^I_{red}. These different

Scheme 2. Redox reactions occurring in the absence of ABzCoA at either ABzCoA-M/R sites E^I or E^{II}, using NADH as electron donor and oxygen or MalNEt as acceptors. Site E^I is where monooxygenation occurs and site E^{II} the one which catalyzes hydrogenation and which is reduced first by NADH. Fast steps, the rates of which have been measured in the following paper are denoted by #.

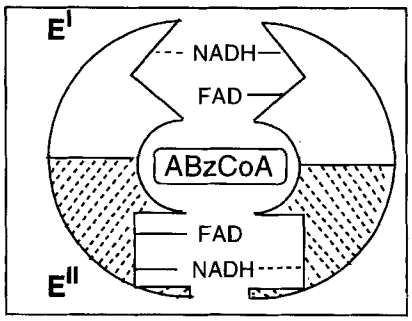


reactivities of sites E^I and E^{II} for oxygen and MalNEt respectively strongly suggest that in catalysis monooxygenation of ABzCoA occurs at site E^I, and is followed by hydrogenation of the non-aromatic intermediate at site E^{II} (compare also to Scheme 2 in Langkau et al., 1990). That this is indeed the case is supported by the results from the reoxidation experiments performed in the presence of substrate ABzCoA, and which are represented by Eqns (3, 4 and 5).

Scheme 2 is also consistent with the reactivities of the three different ABzCoA-M/R species 1, 2, and 3 obtained from chromatography on Mono Q (Fig. 3). Species 1 appears to correspond to fully functional enzyme (containing complete sites E^I and E^{II}); it thus catalyzes monooxygenation and hydrogenation as shown in Scheme 1. Species 3, which is depleted of 50% of total FAD and completely of FAD at site E^{II}, lacks hydrogenation activity. Consequently, during catalysis, the postulated intermediate, ABzOHCoA* (**II**) can only rearomatize to ABzOHCoA (**III**) either on the enzyme surface or spontaneously (Scheme 1).

Based on the report by Altenschmidt et al. (1992) that ABzCoA-M/R is a homodimer and that it contains 1 molecule FAD/monomer, the question arises concerning the mode of formation of two active sites both containing a flavin cofactor, and having different catalytic properties. The fact that FAD interacts in different ways with the protein environment at the two sites, as reflected by the major differences in binding/dissociation properties and, most importantly, reactivity, is consistent with the presence of two distinct catalytic sites. The less likely alternative would be two identical sites which become different upon binding of substrate(s), i.e. a strong negative cooperativity effect. Our interpretation is in line with the finding that there is no exchange of redox equivalents between the two flavin cofactors. This suggests a kinetic barrier since the redox potential measurements with ABzCoA-M/R both in the presence and absence of ABzCoA do not indicate relevant differences between the two flavins. There are probably also two distinct binding sites for the cosubstrate NADH since in the case of a single one, which would have to access both flavins (compare to location

Scheme 3. Hypothetical schematic arrangement of the two polypeptide subunits of ABzCoA-M/R to form two active sites, and mode of binding of flavin cofactor, and of substrates NADH and ABzCoA. The left and right structures represent the two polypeptide chains which interact to form an asymmetric dimer. Its lower and upper parts form the two different active sites E^I (hydroxylation) and E^{II} (hydrogenation). An enzyme in which the two chains fold in two distinct patterns yielding two active centers E^I and E^{II} each and forming a single binding pocket for ABzCoA is also conceivable, albeit less attractive.



of ABzCoA in Scheme 3), one might expect exchange of redox equivalents between the latter. We envisage Scheme 3 as a possible model for rationalizing our findings. In this scheme there is only one binding site for the substrate ABzCoA which must be able to access both sites E^I and E^{II}. Clearly the question about the factors which induce creation of two different active sites using two seemingly identical polypeptide chains is of basic importance. While there are plenty of examples of enzymes in which two or more different cofactors act or participate in acting on a single substrate, a notable one being the Molybdo-FeS-FAD oxidases such as xanthine oxidase, the finding of the same cofactor being used in a single protein for the catalysis of two different chemical reactions is unprecedented to our knowledge. An analogy could be represented by the FAD and FMN cofactors in NADPH-P_{450} reductases (Vermillion and Coon, 1978) or

in dihydroorotate dehydrogenase (Friedmann and Vennesland, 1960; Miller and Massey, 1965), but in these two latter cases one center is assumed to serve in simply electron transfer.

The observation of three enzyme forms separating on ion-exchange chromatography does not exclude the hypothesis that post-translational modifications play a role in the sense that they might be involved in the differentiation of the active sites. The fact that dimer species $\alpha\alpha$, $\alpha\alpha'$, and $\alpha'\alpha'$ all have approximately the same amount of hydrogenation and monooxygenation activities, however, implies that this could not be the only cause. The point should also be stressed that the occurrence of the heteromeric species having different affinities for FAD has probably nothing to do with the existence of a dimer with two different active centers. On-going experiments aimed at expressing the enzyme in different organisms such as *Escherichia coli*, and at the determination of the three-dimensional structure might help elucidate this puzzle.

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