First publ. in: Flavins and flavoproteins : proceedings of the eleventh International Symposium Nagoya (Japan), July 27 - 31, 1993, pp. 259-262

2-AMINOBENZOYL-CoA-MONOOXYGENASE/REDUCTASE, AN ENZYME WITH TWO DISTINCT FUNCTIONS AND ONE ACTIVE CENTER

B. Langkau, S. Ghisla

Faculty of Biology, University, P.O. Box 5560, D-78434 Konstanz, Germany

Introduction

2-Aminobenzoyl-CoA monooxygenase/reductase (ACMR) is a homodimeric enzyme from *Pseudomonas sp.* containing one FAD per subunit [1]; it catalyzes the two following reactions [2]:

$$ACoA + NADH + H^+ + O_2 \longrightarrow A(OH)^{\#}CoA + NAD^+ + H_2O \quad (I)$$

$$A(OH)^{\#}CoA + NADH + H^+ \longrightarrow A(OH)H_2CoA + NAD^+ \quad (II)$$

where ACoA is the physiological substrate 2-aminobenzoyl-CoA, A(OH)#CoA is a non-aromatic intermediate and $A(OH)H_2CoA$ is 2-amino-5-oxocyclohex-1-enecarboxyl-CoA. Reaction (I) is a typical *monooxygenation* while (II) is a *hydrogenation* (reduction).
Recently we have shown that ACMR binds 0.5 equivalents ACoA per enzyme flavin and that the kinetics of flavin reduction with NADH and flavin reoxidation with either oxygen or N-ethylmaleimide (NEM) are strongly biphasic [3]. NEM serves as an artificial electron acceptor [1] for ACMR and is considered to be a substrate analog for the hydrogenation reaction (equation II). In this report we discuss the relationship between these half-site reactivities and the two flavin cofactors involved in catalysis.

Results

Resolution of purified ACMR by chromatography on Mono Q

Purified ACMR can be resolved preparatively into three major (1, 2, 3) and two minor species (1a, 2a) by ionic exchange chromatography on Mono Q (Fig. 1). The three main species differ markedly in their specific activities and in their ratios of absorption A280/450, which reflects the proportion of holo- to apo-protein, i.e. the FAD content. This ratio increases from species 1 to 3 suggesting a decrease of FAD content. The relative FAD contents are approximately (in %): Untreated ACMR ≈ 100; fraction 1 ≈ 78; fraction 2 ≈ 72; fraction 3 ≈ 52. Most importantly, the distribution of products depends on whether ACoA is turned over by ACMR

fractions 1, 2 or 3. Upon separation from NAD^+/NADH and enzyme, product analysis was carried out spectrophotometrically as shown in Fig. 2. From the results it is apparent that turnover of ACoA using untreated ACMR yields predominantly $A(OH)H_2CoA$ (IV), while with fraction 3 the content of A(OH)CoA (III) is ≈ 60%. This suggest that all three species 1, 2, and 3 are fully competent in monooxygenation, but vary in their capacity to hydrogenate. The relative differences between these activity profiles can readily be explained by selective loss of FAD at the active site which catalyzes hydrogenation.

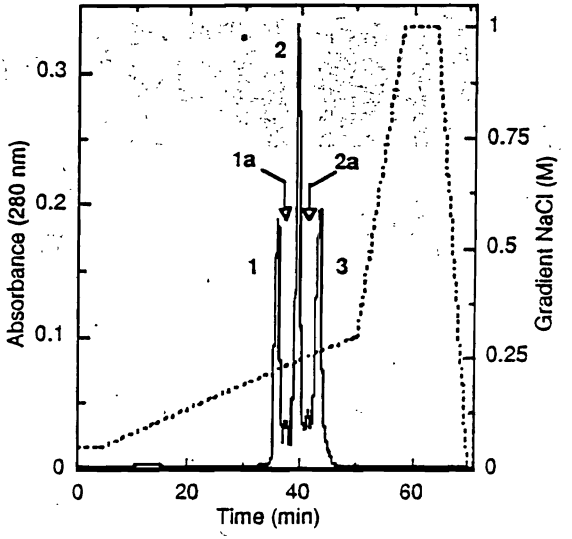


Figure 1. Resolution of purified ACMR by chromatography on MonoQ (experimental details to be published [4])

Kinetics of reoxidation of (half)reduced, uncomplexed enzyme with either O_2 or NEM

The course of reoxidation of fully (photo)reduced, uncomplexed enzyme is strongly biphasic, and the ratio of the extent of the fast compared to that of the slow phase (k_{obs} = 0.09 min^{-1}) is ≈ 1:1. When ACMR is reduced to ≈ 50% and subsequently exposed to oxygen, a monophasic and *slow* reaction (same rate as above) is observed. These results are compatible with the presence of two sites which have different reactivities towards oxygen and are independent from each other. When completely reduced enzyme is reacted anaerobically with NEM qualitatively the same biphasic reaction is observed as with oxygen. However, the reaction of 50%-reduced enzyme with NEM is *fast*, and it corresponds to the fast rather than to the slow phase of the NEM-reaction with fully reduced enzyme. This suggests the presence of two complementary sites. Furthermore, and in agreement with this interpretation, reoxidation of fully reduced enzyme with NEM and subsequent addition of oxygen to the partially reoxidized enzyme leads to fully oxidized enzyme in two fast steps.

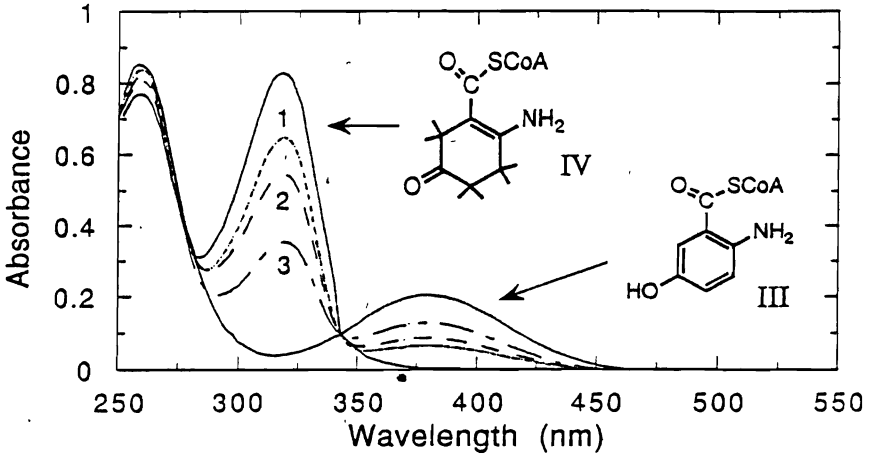
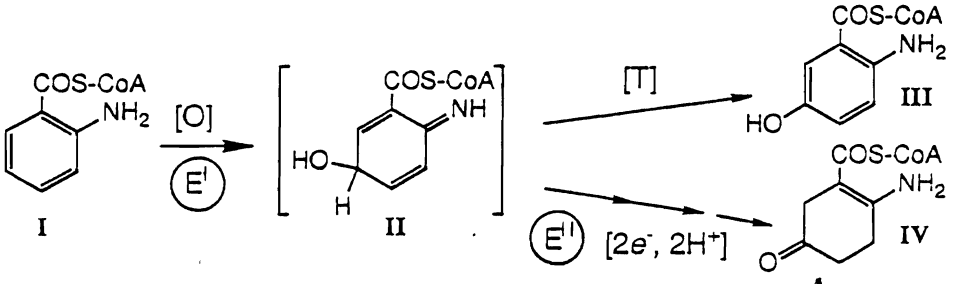


Figure 2: Absorption spectra of product mixtures resulting from incubations of 2-amino-benzoyl-CoA with either untreated ACMR (curve 1), species 1 (curve 2) or species 3 (curve 3) (cf. figure 1). Spectra of the pure products were superimposed for comparison (full lines).

Similar experiments were carried out using species 1, 2 and 3 (see Fig. 1), which have a varying content of FAD cofactor. The results showed that in the reaction of fully reduced enzyme with O_2 the ratio of the fast and slow phase correlate inversely with the FAD content. This corresponds to the ratio of the products A(OH)CoA vs. 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 ACMR has two different active sites, one which catalyzes the monooxygenation and reacts fast with oxygen, and the other one which catalyzes the hydrogenation and reacts slowly with oxygen but fast with the substrate (product) analog NEM.

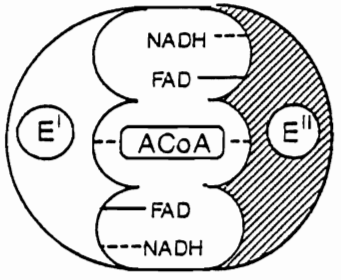


Scheme 1: Reactions catalyzed by ACMR. At the "monooxygenation" site E^I oxygen insertion in the substrate I (reaction [O]) occurs to form intermediate II. In the case of functional E^{II}, the latter is hydrogenated quickly at this site to produce a tautomeric precursor of the final product IV. When E^{II} is not functional spontaneous tautomerization (T) to the aromatic III occurs.

Conclusions

Scheme 1 is consistent with the reactivities of the three different ACMR species 1, 2, and 3 obtained from chromatography on Mono Q (Fig. 1). Untreated ACMR corresponds functional enzyme and possesses complete sites E^{I} and E^{II}; it catalyzes monooxygenation (O) and hydrogenation ($2H^{+}$, $2e^{-}$) as shown in scheme 1. Fraction 3, which is depleted of ≈ 50% the total FAD corresponding to that of the FAD of site E^{II}, lacks hydrogenation activity. Consequently, during catalysis, the postulated intermediate A(OH)#CoA (II) rearomatizes to A(OH)CoA (III) either on the enzyme surface or spontaneously (Scheme 1). Fraction 1 and 2 are intermediate cases.

Since ACMR is a homodimer [1] the question arises how two structurally and functionally distinct active sites, both containing a flavin cofactor, are formed. 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, 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). Our interpretation is in line with the finding that the exchange of redox equivalents between the two flavin cofactors is at best slow. This suggests a kinetic barrier since the redox potential measurements with ACMR both in the presence and absence of ACoA do not indicate 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, one might expect exchange of redox equivalents between the latter. Finally, the model of Scheme 2 also has only one binding site for the aromatic substrate ACoA, and the latter must be able to access both sites E^{I} and E^{II}.



Scheme 2: Proposed schematic assembly of ACMR subunits E^{I} and E^{II} to form two active centers both having access to substrate ACoA and both containing one NADH and one FAD binding sites.

Acknowledgements:
This work was supported by grant Gh 2/4-7 from the Deutsche Forschungsgemeinschaft.

References

1. Buder, R. and Fuchs, G. (1989) Eur. J. Biochem. 185, 629-635
2. Langkau, B., Ghisla, S., Buder, R., Ziegler, K., and Fuchs, G. (1990), Eur. J. Biochem. 191, 365-371
3. Langkau, B., Ghisla, S., Massey, V., and Fuchs, G., (1990) in "Flavins and Flavoproteins" W. deGruyter, Berlin, New York, pp. 239-242
4. Langkau, B., Vock, P., Fuchs, G., and Ghisla, S., in preparation.