Biochimica et Biophysica Acta 1854 (2015) 890–896

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbapap

Anthranoyl-CoA monooxygenase/reductase from *Azoarcus evansii* possesses both FMN and FAD in two distinct and independent active sites

Thomas Bergner [a], Tea Pavkov-Keller [b], Katharina Kreuzer [a], Jakob Kowaliuk [a], Markus Plank [a], Kathrin Runggatscher [a], Nikolaus G. Turrini [c], Benjamin Zucol [a], Silvia Wallner [a], Kurt Faber [c], Karl Gruber [d], Peter Macheroux [a,*]

[a] *Institute of Biochemistry, Graz University of Technology, A-8010 Graz, Austria*
[b] *ACIB-Austrian Centre of Industrial Biotechnology, A-8010 Graz, Austria*
[c] *Institute of Chemistry, University of Graz, A-8010 Graz, Austria*
[d] *Institute of Molecular Biosciences, University of Graz, A-8010 Graz, Austria*

ARTICLE INFO

Article history:
Received 12 January 2015
Received in revised form 10 March 2015
Accepted 26 March 2015
Available online 2 April 2015

Keywords:
Flavin
Monooxygenase
Reductase
Old Yellow Enzyme
Microcalorimetry
Small-angle X-ray scattering

ABSTRACT

Anthranoyl-CoA monooxygenase/reductase (ACMR) participates in an unusual pathway for the degradation of aromatic compounds in *Azoarcus evansii*. It catalyzes the monooxygenation of anthranoyl-CoA to 5-hydroxyl-2-aminobenzoyl-CoA and the subsequent reduction to the dearomatized product 2-amino-5-oxo-cyclohex-1-ene-1-carbonyl-CoA. The two reactions occur in separate domains, termed the monooxygenase and reductase domain. Both domains were reported to utilize FAD as a cofactor for hydroxylation and reduction, respectively. We have heterologously expressed ACMR in *Escherichia coli* BL21 and found that the monooxygenase domain contains FAD. However, the reductase domain utilizes FMN and not FAD for the reduction of the intermediate 5-hydroxyl-2-aminobenzoyl-CoA. A homology model for the reductase domain predicted a topology similar to the Old Yellow Enzyme family, which exclusively bind FMN, in accordance with our results. Binding studies with 2-aminobenzoyl-CoA (AbCoA) and *p*-hydroxybenzaldehyde (pHB) as probes for the monooxygenase and reductase domain, respectively, indicated that two functionally distinct and independent active sites exist. Given the homodimeric quartenary structure of ACMR and the compact shape of the dimer as determined by small-angle X-ray scattering experiments we propose that the monooxygenase and reductase domain of opposite peptide chains are involved in the transformation of anthranoyl-CoA to 2-amino-5-oxo-cyclohex-1-ene-1-carbonyl-CoA.

© 2015 Elsevier B.V. All rights reserved.

1. Introduction

2-Aminobenzoic acid (anthranilic acid) is an important intermediate in the synthesis and degradation of many *N*-heterocyclic compounds such as tryptophan. As a consequence of its wide occurrence, 2-aminobenzoate is a common substrate for many microorganisms that are able to cleave aromatic rings. *Azoarcus evansii* has developed an unusual way for the degradation of this compound, where typical intermediates like catechol or gentisate were not observed. In contrast to the other known pathways the degradation is linked to the activation of the substrate by an initial formation of a CoA-thioester. The first two enzymes involved in the degradation of 2-aminobenzoate to a nonaromatic product are the 2-aminobenzoate-CoA ligase and 2-aminobenzoyl-CoA (AbCoA) monooxygenase/reductase (ACMR). ACMR is a bifunctional flavoenzyme catalyzing both monooxygenation and reduction of 2-aminobenzoyl-CoA requiring 2 NADH and one O_2 [1,2].

The first reaction catalyzed by the homodimeric ACMR is a nucleophilic attack of the $C_{(4a)}$-hydroperoxyflavin, formed in the active site of the monoxygenase domain and transfer of an oxygen atom to position 5 of the activated substrate. Subsequently the intermediate is transferred to the reductase domain, where a hydride transfer leads to the formation of the nonaromatic cyclohexene product. The overall reaction strongly depends on the presence of NADH. If NADH is limiting no reduction of the intermediate occurs and instead re-aromatization is observed [3].

The N-terminal monooxygenase domain of the protein (amino acids 1–362) showed high similarity to FAD-dependent bacterial hydroxylases [2], whereas the C-terminal domain (403–773) shows similarity to NADH-dependent oxidoreductases and the FMN-dependent family of old yellow enzymes (*e.g.* OYE from *Saccharomyces cerevisiae*) [2]. Interestingly, a previous study suggested the presence of two molecules of FAD/ACMR, *i.e.* both the predicted monooxygenase and reductase (OYE) domain contain FAD. This appeared to be very unusual because all flavoenzymes adopting an $(\alpha\beta)_8$-barrel structure ("TIM"-barrel), including the OYE family, have a strict preference for FMN rather than FAD. To analyze the flavin content of ACMR, we have expressed the

* Corresponding author at: Graz University of Technology, Institute of Biochemistry, Petersgasse 12, A-8010 Graz, Austria. Tel.: +43 316 8736450; fax: +43 316 8736952.
E-mail address: peter.macheroux@tugraz.at (P. Macheroux).

http://dx.doi.org/10.1016/j.bbapap.2015.03.011
1570-9639/© 2015 Elsevier B.V. All rights reserved.

encoding gene in *E. coli* BL21 and purified ACMR to homogeneity for biochemical and structural studies. This enabled us to demonstrate that ACMR contains approximately equal amounts of both FMN and FAD. Furthermore, separate expression of the monooxygenase and reductase domain showed that the reductase domain binds FMN whereas the monooxygenase has FAD as cofactor.

We have also exploited the property of OYEs to bind phenolic ligands to the active site [4], such as *p*-hydroxybenzaldeyde (pHB), to investigate the interdependence of the FAD binding site in the monooxygenase and the FMN binding site in the OYE domain. Towards this end, we have performed difference absorption titrations as well as isothermal titration microcalorimetry measurements to show that the two domains independently bind AbCoA and pHB. This suggests that the CoA moiety of the substrate binds in a central position to enable consecutive processing of the anthranoyl-moiety (first) in the monooxygenase and (second) in the reductase domain.

2. Materials & methods

2.1. Construction of the expression plasmid for ACMR, ACM and ACR

Based on the reported DNA sequence found in operon II, a synthetic gene of the ACMR was designed and optimized for expression in *E. coli* (DNA 2.0, CA, USA). Furthermore it contains a C-terminal octa-histidine tag and a TEV-protease cleavage site. The synthetic DNA was integrated into the vector pET-21a(+) or pET-28a using the restriction sites *Nde*I and *Xho*I, allowing the use of the N-terminal hexa-histidine tag of the vector (pET-28a) if required for facilitated protein purification by Ni-NTA affinity chromatography. *E. coli* BL21 (DE3) was used for heterologous expression.

For the expression of the separated subunits, either site directed mutagenesis or common PCR with primer overhang was performed (sequences of the used primers are summarized in supplementary Table S1) in a way that the construct contained a *Nde*I restriction site at the 5′- and a *Xho*I site at the 3′-end. Both subunits were expressed with and without the linker region. *E. coli* BL21 (DE3) and *E. coli* Rosetta (DE3) were used for heterologous expression of ACM and ARC, respectively.

2.2. Expression and purification of recombinant His-tagged proteins in E. coli

E. coli cells harboring the expression plasmids were grown at 37 °C in LB broth and depending on the used expression plasmid either ampicillin (100 μg/ml) (for pET-21a) or kanamycin (50 g/ml) (for pET-28a) was used as selection marker. The cells were induced with 0.5 mM IPTG at $OD_{600} = 0.6$. After induction the cells were further grown for 16 h at 20 °C. Cells were harvested by centrifugation (7000 *g*, 10 min, at 4 °C) and the cell pellet was stored at −20 °C for further use.

For purification of the recombinant proteins, the cell paste was re-suspended in lysis buffer pH 8 (50 mM $NaH_2PO_4 \cdot H_2O$, 300 mM NaCl and 10 mM imidazole) or pH 8.5 for ACR and lysed by sonication. To remove cell debris the resulting suspension was centrifuged at 30,000 *g* for 45 min at 4 °C, followed by an additional filtration step. The cleared solution was then loaded onto a pre-equilibrated 5 ml HisTrap FF column (GE Healthcare), washed with about 10 column volumes of wash buffer (50 mM NaH_2PO_4, 300 mM NaCl and 20 mM imidazole) and finally eluted with elution buffer (50 mM $NaH_2PO_4 \cdot H_2O$, 300 mM NaCl and 150 mM imidazole). Protein containing fractions were pooled and dialyzed against 20 mM Tris buffer containing 100 mM NaCl, pH 8 or pH 8.5 for ACMR and ACM or ACR, respectively. After concentration the proteins were further purified using a Superdex-200 gel filtration column equilibrated with dialysis buffer. The purified protein was concentrated and stored at −20 °C. The concentrations were determined spectrophotometrically. The molar extinction coefficient for ACMR and ACM was calculated yielding 21,500 M^{-1} cm^{-1} (459 nm) and 10,130 M^{-1} cm^{-1} (450 nm) [5]. The molar extinction coefficient at 280 nm of apo-ACR ($\varepsilon = 57{,}785$ M^{-1} cm^{-1}) was calculated employing ProtParam at the ExPASy site following the method of Gill and von Hippel [6]. The amount of isolated protein obtained from 1 l culture was about 25 mg, 30 mg and 15 mg for ACMR, ACM and ACR, respectively.

2.3. Catalytic activity of recombinant ACMR

The rate of reduction of ACMR by NADH was determined by stopped-flow absorption spectrophotometry under anoxic conditions using a stopped-flow device from Hi-Tech, Bradford-on-Avon, UK (model SF-61DX2). All experiments were performed in 50 mM sodium phosphate buffer, pH 7.5 at 4 °C. Catalytic competence of the reductase domain was tested using 2-methyl-*N*-phenylmaleimide as a model substrate using a 1.5-fold excess of NADH in 50 mM Tris/HCl-buffer, pH 7.5, at 30 °C.

2.4. Determination of flavins

For cofactor determination concentrated protein samples were heated up to 95 °C and the precipitated protein was removed by centrifugation (7000 *g*, 5 min, at 4 °C). The supernatant was subsequently concentrated in a speed vac at 50 °C under reduced pressure and analyzed by HPLC.

HPLC analysis was performed with a Dionex UltiMate 3000 HPLC, Thermo Scientific (Waltham, MA, USA) using an Atlantis® dC18 5 μM (4.6 × 250 mm) column. As liquid phase a 0.1% TFA solution and acetonitrile containing 0.1% TFA were used. The concentration of the organic solvent was increased from 0% to 95% using a linear gradient over 20 min. During the whole time the temperature was adjusted to 25 °C and flow rate was set to 1 ml/min. The samples were analyzed using a diode array detector, the detection wavelengths were set to 280, 370 and 450 nm, respectively.

2.5. Homology modeling

Structural models of both domains (residues 1–339 and 405–755) were generated using the Phyre2 [7] and Swiss-Model [8] servers. The structures of the OYE from *Thermus scotoductus* (PDB-entry: 3HGJ, [9]) and of VioD hydroxylase from *Chromobacterium violaceum* (PDB-entry: 3C4A, Northeast Structural Genomics Consortium, unpublished) were used as templates for the individual domains. Sequence identities were 34% (for the OYE domain) and 32% (for the hydroxylase domain).

2.6. Small-angle X-ray scattering (SAXS)

Small angle X-ray scattering data were collected on the X33 EMBL beam line at the storage ring DORIS III (DESY, Hamburg, Germany) [10]. The data were recorded using a MAR345 two-dimensional imaging plate detector at a sample-detector distance of 2.7 m and a wavelength of $\lambda = 0.15$ nm, covering the range of momentum transfer $0.12 < s < 4.5$ nm^{-1} ($s = 4\pi \sin\theta/\lambda$, where 2θ is the scattering angle). Data processing was performed using the program package PRIMUS [11]. The ACMR protein solution (in 10 mM Tris/HCl buffer pH 8.0) was measured at three different concentrations: 3.6, 6.7 and 14.8 mg/ml. Data obtained at the low and high concentration were cut accordingly and merged for subsequent data analysis. The forward scattering *I(0)*, the radius of gyration (R_g) and the maximum diameter D_{max} were evaluated using the Guinier approximation [12] and the program GNOM [13]. A solution of bovine serum albumin (molecular mass = 66 kDa) at 5 mg/ml in water was used as a reference. The *ab initio* shape reconstruction was performed using the program GASBOR [14]. The results from at least ten separate GASBOR runs were averaged to determine common structural features using the programs SUPCOMB [15] and DAMAVER [16].

Download English Version:

https://daneshyari.com/en/article/1177796

Download Persian Version:

https://daneshyari.com/article/1177796

Daneshyari.com