Appl Biochem Biotechnol (2011) 165:823–831
DOI 10.1007/s12010-011-9300-5

An Improved Procedure for the Purification of Catalytically Active Alkane Hydroxylase from *Pseudomonas putida* GPo1

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Received: 8 December 2010 / Accepted: 26 May 2011 /
Published online: 7 June 2011

Abstract Bacterial alkane hydroxylases are of high interest for bioremediation applications as they allow some bacteria to grow in oil-contaminated environments. Furthermore, they have tremendous biotechnological potential as they catalyse the stereo- and regio-specific hydroxylation of chemically inert alkanes, which can then be used in the synthesis of pharmaceuticals and other high-cost chemicals. Despite their potential, progress on the detailed characterization of these systems has so far been slow mainly due to the lack of a robust procedure to purify its membrane protein component, monooxygenase AlkB, in a stable and active form. This study reports a new method for isolating milligramme amounts of recombinant *Pseudomonas putida* GPo1 AlkB in a folded, catalytically active form to purity levels above 90%. AlkB solubilised and purified in the detergent lauryldimethylamine oxide was demonstrated to be active in catalysing the epoxidation reaction of 1-octene with an estimated K_m value of 0.2 mM.

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Keywords Biocatalysis · Alkane hydroxylase · Biomodification of hydrocarbons · Alkane biodegradation

Introduction

The alkane hydroxylase systems found in many bacteria allow them to use hydrocarbons as the sole source of carbon and energy, a natural process of great potential for the bioremediation of oil-contaminated environments [1]. These enzymes catalyse the energetically demanding hydroxylation of terminal methyl groups, the first step in the catabolic breakdown of alkanes. This same reaction is of great biotechnological interest, as it leads to the chemical functionalisation of otherwise inert alkanes under conditions not achievable by metal or organometallic catalysts [2, 3]. Therefore, there is a strong incentive to study these systems in great detail.

The most well-characterised alkane hydroxylase system to date is that of *Pseudomonas putida* GPo1 [4–7]. It comprises three different proteins: a soluble NADH–rubredoxin reductase, a soluble electron-transfer protein rubredoxin and an integral cytoplasmic membrane mooxygenase AlkB, usually referred to as alkane hydroxylase. AlkB transfers one oxygen atom from O_2 to the alkane molecule, producing an alcohol, while the other oxygen is reduced to H_2O by the electrons transferred from NADH via rubredoxin reductase and rubredoxin. Besides catalysing the regio- and stereo-selective hydroxylation of aliphatic and alicyclic compounds, the alkane hydroxylase system can oxidize terminal alcohols to the corresponding aldehydes, demethylate branched methyl ethers, sulfoxidate thioethers and epoxidate terminal olefins and allyl alcohol derivatives [6, 8–11]. It is one of a few alkane hydroxylases that can oxidize C3 to C12 n-alkanes [12], with maximal conversion rates for octane and octene [8, 13]. Although the biocatalytic potential of alkane hydroxylase AlkB is well recognised, little is known about the mechanism of the catalysis, the spatial arrangement of the catalytic residues [5, 14] and the substrate-binding pocket [6], mainly because our understanding of its structure is limited to an inferred folding topology [15].

Progress on the detailed characterisation of AlkB has so far been slow due to the lack of a robust procedure to purify this membrane protein in a stable and active form. Not many membrane proteins remain structurally and functionally sound after extraction from their native lipid environment, and therefore, it is always challenging to find the right conditions under which a detergent-solubilised membrane enzyme is stable and catalytically active. Accordingly, it is not surprising that all studies conducted so far on AlkB have been limited to whole cells, cell extracts or partially purified enzyme [12, 14, 16–21]. Although adequate for some analysis, these crude enzymatic preparations are not appropriate for structural studies or detailed catalytic characterization. Not only could the contaminant proteins result in the unwanted metabolism of the hydroxylated product but also the existence of multiple alkane hydroxylase genes with overlapping substrate ranges within a single organism further constrains such approaches.

To address some of the issues that have so far hindered the detailed investigation of the structure, reaction kinetics and mechanism of AlkB, this paper introduces a new method for the isolation of milligramme amounts of detergent-solubilised recombinant *P. putida* GPo1 AlkB in a folded, catalytically active form to purity levels above 90% and presents a generic in vitro activity assay that allows testing of a range of water-insoluble hydrocarbon substrates in the presence of detergent micelles.

Materials and Methods

Bacterial Strains and Plasmids

The pGEc47 plasmid [22] was purchased from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH). *Escherichia coli* Novablue and Tuner(DE3) pLacI strains and pETBlue-2 vector were purchased from Novagen.

Construction of the Recombinant Plasmid

The pGEc47 plasmid harbouring the *P. putida* GPo1 *alkB* gene was used as a template for PCR amplification of the *alkB orf*. The gene sequence was extended by adding the StrepII tag (EMBL reference number: CAB70506) to the C terminus via a two-step PCR using Phusion DNA polymerase (Finnzyme). The first step PCR was performed using primers PpAlkBF (5′-acttgagaaacacagagttctggat-3′, forward) and PpAlkBR1 (5′-gtggctccaagcgctcgatgctaccgcagaggta-3′, reverse). The PCR product was gel-purified using QIAquick Gel Extraction Kit (Qiagen) and used as a template for the second step PCR with primers PpAlkBF and PpAlkBR2 (tttttcgaactgcgggtggctccaagcgct). The PCR was performed as follows: 98 °C for 5 min, followed by 35 cycles at 98 °C for 30 s, 61 °C for 30 s and 72 °C for 18 s, followed by a final extension at 72 °C for 10 min. The second step PCR product was gel-purified and cloned into the pETBlue-2 vector using the Perfectly Blunt Cloning Kit (Novagen) to produce the expression vector pBlue2-AlkBStrepII. The ligation product was then transformed into competent *E. coli* NovaBlue cells. Recombinant clones harbouring the *alkB-StrepII* fragment were selected by blue/white screening. Plasmids were isolated using the QIAprep Spin Miniprep Kit (Qiagen). DNA was quantified using Nanodrop® ND-1000 spectrophotometer. The expression clone was confirmed by DNA sequencing.

Over-expression of Recombinant AlkB and Isolation of the Membrane Fraction

To express recombinant AlkB with a C-terminal StrepII tag (AlkB-StrepII), the plasmid pBlue2-AlkBStrepII was transformed into Tuner(DE3)pLacI competent cells. Cells were grown in 10 L of LB medium containing 50 μg carbenicillin per millilitre and 34 μg chloramphenicol per millilitre at 37 °C with shaking at 200 rpm to an OD_{600} of 0.6–0.8. The expression of recombinant AlkB was induced at that point by adding 1 mM isopropyl-β-D-thio-galactoside (IPTG), and growth continued for further 3 h. The cells were then harvested by centrifugation at 4,500×*g* for 20 min at 4 °C.

To isolate the membrane fraction, the cell paste was resuspended in three times its volume of a buffer containing 150 mM NaCl, 50 mM Tris/HCl pH 7.4 and 1 mM phenylmethylsulphonyl fluoride (PMSF) and sonicated for 10 min with the TT13 probe (Bandelin) at 40% amplitude on a 10-s on and 10-s off cycle. The cell lysate was first spun down at 10,000×*g* for 40 min at 4 °C to precipitate cell debris, and the supernatant was further spun down at 120,000×*g* for 2 h at 4 °C to pellet the membranes containing the protein of interest (AlkB).

Detergent Solubilisation of the Membrane Fraction

Membranes were solubilised in 50 ml of buffer NP (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 1 mM PMSF) supplemented with 10 critical micelle concentration (CMC)

of detergent by stirring at 4 °C for 1 h. The detergents tested were 0.23% lauryldimethylamine oxide (LDAO, Fluka), 0.87% *n*-decyl-β-D-maltopyranoside (DM, Anatrace), 0.15% polyoxyethylene(8)dodecyl ether ($C_{12}E_8$, Anatrace) and 5.3% *n*-octyl-β-D-glucopyranoside (OG, Anatrace). After membrane solubilisation, the solution was diluted fivefold with the NP buffer to avoid protein denaturation in high detergent concentration and centrifuged at 120,000×*g* for 30 min to pellet undissolved membranes.

Western Blotting Analysis

Western blotting analysis was performed using the monoclonal anti-StrepII antibody (Novagen) to visualize and quantify the recombinant AlkB-StrepII. For dot-blot analysis, 15 μg of the total solubilised membrane protein was spotted onto a nitrocellulose membrane, which was dried and processed according to the Western-Breeze chemiluminescent immunodetection protocol (Invitrogen). Proteins separated on a 12% (*v/v*) sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) gel were electrophoretically transferred onto nitrocellulose membrane in the transfer buffer containing 25 mM Tris, 192 mM glycine and 20% (*v/v*) methanol, under 65 V for 2 h, and the membrane processed as above.

Purification of Recombinant *P. putida* AlkB and Rubredoxin

The solubilised membrane fraction was incubated with the Strep-Tactin MacroPrep Resin at a ratio of 100 μl resin per 25 ml of solubilised membranes with rolling at 4 °C for 16 h. The resin was pelleted by centrifugation at 1,500×*g* for 15 min and washed in the NP buffer supplemented with 0.4–1.5 mM D-desthiobiotin (DTB) and 0.07% (3CMC) LDAO for 10 min with gentle stirring. AlkB was eluted with the NP buffer containing 2.5 mM DTB and 0.07% LDAO. Recombinant *P. putida* rubredoxin was expressed from the pKR10 plasmid in *E. coli* TG10 cells and purified as described [23]. The protein concentration was determined using Bradford assay [24], and the purity of the samples was assessed using 12% SDS-PAGE.

Enzyme Assay

The hydroxylase activity of the purified recombinant AlkB was measured by following a modified protocol of McKenna and Coon [16]. A typical reaction mixture contained 100 mM Tris/HCl pH 7.4, 0.035% LDAO (1.5 CMC), 20% (*v/v*) glycerol, 3 μM rubredoxin, 0.25 μM rubredoxin reductase, 0.1–1.0 mM 1-octene (Sigma) and 3–9 μM AlkB. The mixture was incubated at 30 °C for 2 min, after which the reaction was initiated by adding NADH (Melford) to a final concentration of 50 μM. The rate of NADH consumption was determined by monitoring the change in absorbance at 340 nm at room temperature for 5 min using the Cary 300 Scan UV–visible spectrophotometer.

Circular Dichroism

Far-UV circular dischroism (CD) spectra were recorded for recombinant AlkB at 1 mg/ml at 20 °C using a JASCO J600 spectrapolarimeter (calibrated with 0.06% d-10 camphorsulphonic acid) over the wavelength range 190–260 nm in a quartz cylindrical cell of 0.02 cm pathlength (scan rate of 10 nm min^{-1}). Spectra were recorded in triplicate and averaged. The samples were in buffer NP supplemented with 0.07% LDAO. The

percentage of secondary structure was calculated by deconvoluting the circular dichroism spectra using the online K2d CD secondary structure server (www.embl-heidelberg.de/~andrade/k2d/) [25].

Results

Cloning of the *alkb* Gene and Optimization of the Expression Conditions

To facilitate the subsequent protein purification via affinity chromatography, the gene encoding *P. putida* AlkB was cloned with a C-terminal Strep II tag into the pETBlue-2 vector. Expression conditions were optimized to yield the highest levels of AlkB in the membrane fraction as judged by Western blot analysis of SDS-PAGE gels (not shown). The best levels of membrane-integrated AlkB were achieved when the Tuner(DE3)pLacI cells transformed with the pBlue2-AlkBStrepII plasmid were grown at 37 °C and protein expression was induced with 1 mM IPTG for 3 h at the same temperature. The recombinant protein migrated on SDS-PAGE gels with an apparent molecular weight of 44–46 kDa (Fig. 1), which is close to the value calculated from the amino acid sequence (46 kDa).

Selection of Detergent for Solubilisation of AlkB and Protein Purification

The membranes fraction containing recombinant AlkB was solubilised with 10 CMC of four different detergents commonly used for biological membrane solubilisation (LDAO, DM, $C_{12}E_8$ and OG). Unsolubilised material and large aggregates were removed by centrifuging at 120,000×*g*, and the relative amount of solubilised AlkB was assessed by SDS-PAGE analysis followed by Western blotting with the anti-StrepII antibody. The yield of AlkB in the solubilisation step was highest with LDAO (data not shown). Therefore, LDAO was selected for solubilisation and subsequent purification of AlkB.

Recombinant AlkB was isolated using one-step Strep-Tactin affinity batch purification from the solubilised membranes of *E. coli*. To find an optimal concentration of D-desthiobiotin (DTB) in the wash step, the AlkB-bound resin was washed with the NP buffer containing 0.07% LDAO and four different DTB concentrations (0.4, 1, 1.5 and 0 mM DTB, respectively), after which the protein was eluted with the same buffer containing 2.5 mM DTB and its purity analysed by SDS-PAGE. Including 1 mM DTB in the wash step resulted in a >90% purity of eluted AlkB (Fig. 1). Approximately 1 mg of pure AlkB was obtained from 10 L of bacterial culture.

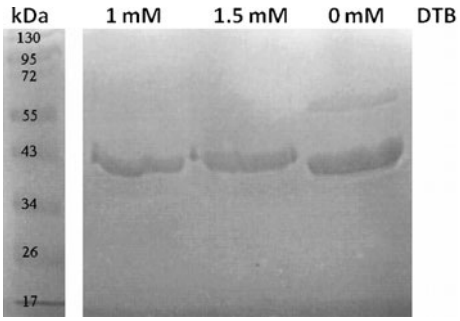

Fig. 1 Coomassie blue-stained SDS-PAGE gel of purified AlkB-StrepII eluted from Strep-Tactin MacroPrep resin following wash with the NP buffer containing 0.07% mM LDAO and 1, 1.5 and 0 mM DTB, respectively. In each lane, 15 μg of eluted AlkB was loaded

Assessing Activity of Detergent-Solubilised AlkB

Circular dichroism was used to investigate the secondary structure of the recombinant *P. putida* AlkB protein (Fig. 2). Estimation of the α-helix and β-sheet content in the secondary structure using the K2d server [25] gave the values (α 44%, β 19%) that are very close to those predicted from the sequence analysis using the Jpred server [26] (α 51%, β14%), indicating that the LDAO-solubilised protein is folded.

A spectrophotometric assay was designed to characterise the alkane hydroxylase-catalysed epoxidation of 1-octene at various concentrations of AlkB and the substrate. To determine the initial reaction rate, NADH to NAD^+ conversion was monitored by measuring the change in absorbance at 340 nm after the addition of AlkB and the substrate (Fig. 3). Three control reactions (lacking AlkB, lacking the substrate and lacking both AlkB and the substrate) were included to measure the background reaction rate. In the absence of both AlkB and the substrate, no measurable NADH oxidation was detected. In the absence of AlkB and a presence of 0.1 mM 1-octene, there was an initial drop in absorbance at 340 nm in the first 2.5 min at a rate of 0.03 AU/min with no change observed afterwards (Fig. 3a). The rubredoxin reductase component of the alkane hydroxylase system transfers the electron from NADH to rubredoxin, which subsequently donates it to AlkB. Therefore, the initial drop in absorbance in the absence of AlkB can be attributed to NADH being oxidized by rubredoxin reductase. In the absence of substrate and the presence of 3 μM AlkB, a background NADH consumption was observed, with an initial rate of 0.14 AU/min. This background activity is likely to be due to the activity of contaminating NADH–reductase(s) co-purified with AlkB.

As shown in Figs. 3 and 4, increasing the concentration of 1-octene resulted in an increase in the initial reaction rate. The initial reaction rates at various substrate concentrations were plotted to estimate the Michaelis constant (K_m) of AlkB for 1-octene. This analysis yielded an estimated value for K_m of 0.2 mM, which is near the range of values previously reported for activity of a partially purified AlkB with hexanoate (22 mM), heptanoate (5.2 mM) and nonanoate (0.69 mM) [16].

Discussion

Despite its tremendous biotechnological potential as a biocatalyst and promising application in the bioremediation of oil-contaminated environments, very little is known about the

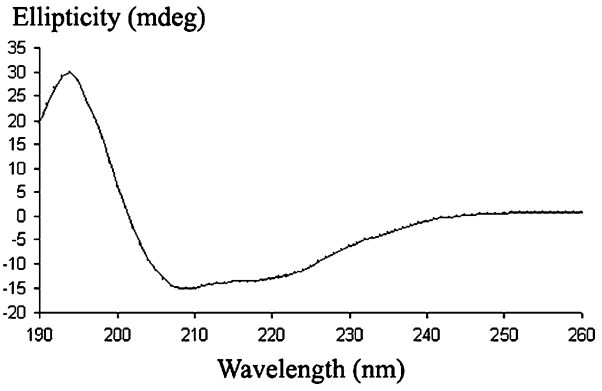

Fig. 2 Circular dichroism spectra of recombinant *P. putida* AlkB. Molar ellipticity in the far-UV range (190–260 nm) is plotted for AlkB at 1 mg/ml. Analysis of this data using the K2d server predicts that the secondary structure of AlkB is 44% α-helical, 19% β-sheet and 37% random coil

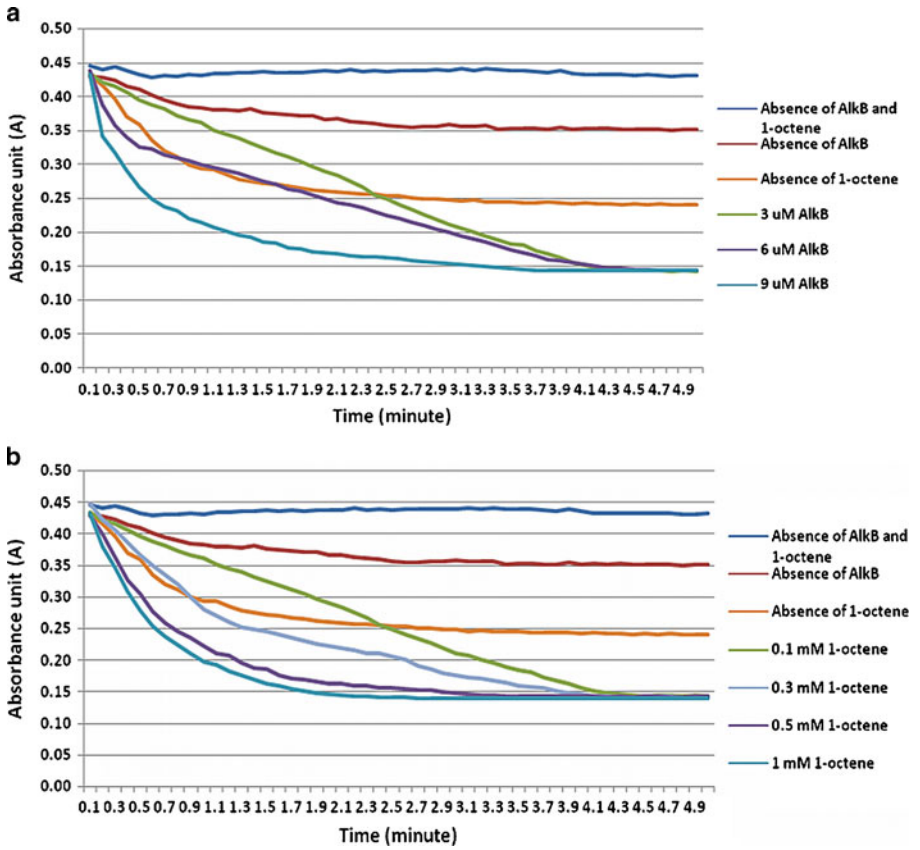

Fig. 3 Characterization of the epoxidation reaction catalysed by AlkB. UV–visual absorption spectra change at 340 nm for the 1-octene epoxidation reaction catalysed by AlkB at various AlkB concentrations with the same substrate concentration (0.1 mM) (**a**) and various substrate concentrations with the same AlkB concentration (3 μM) (**b**)

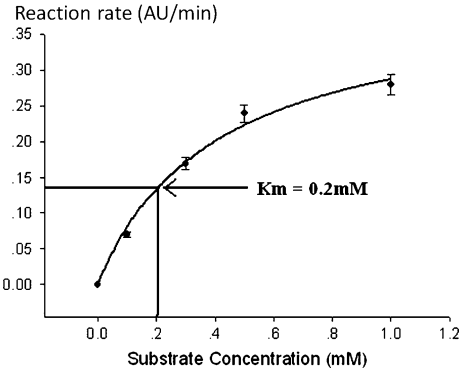

Fig. 4 Estimation of the Michaelis constant (K_m) of AlkB for 1-octene. The initial reaction rates at substrate concentrations of 0.1, 0.3, 0.5 and 1 mM were plotted to estimate the K_m value of AlkB for 1-octene. K_m is defined as the concentration of substrate at which the reaction rate is at half of the maximum rate. The estimated K_m value for 1-octene is approximately 0.2 mM. The *line* through the data points is the fit for a single rectangular hyperbola $y = ax/(b + x)$. The *error bars* represent the range of values for three independent experiments. The graph was plotted with SigmaPlot 2000

structure–function relationship of AlkB. Structural and kinetic studies have been limited so far to whole cells or partially purified proteins. For the first time, we present a simple procedure to purify catalytically active AlkB in milligramme amounts and at purity levels greater than 90%. The recombinant enzyme was expressed in *E. coli* cells, extracted from the membrane fraction using the detergent LDAO and purified using affinity chromatography. Thanks to the high specificity of the strep tag and the detergent extraction of AlkB, very high purity was achieved in just one purification step. The structural quality of the purified protein was confirmed by circular dichroism analysis, which resulted in α-helix and β-sheet contents (α 44%, β 19%) very close to the predicted values (α 51%, β 14%). Furthermore, our kinetic analysis also showed that the purified AlkB was active, capable of catalysing the epoxidation reaction of 1-octane. The presence of the LDAO detergent in the spectrophotometric assay facilitated the solubilisation of the hydrophobic 1-alkene, providing an improved setup to those previously used, which had to include the detergent Triton X-100 to solubilise the substrates [4, 16], with unknown effects on the stability/function of AlkB.

The present paper addresses some of the key issues that have so far hindered the detailed investigation of the integral membrane monooxygenase AlkB. We have developed a quick and reliable purification method that yields catalytically active recombinant *P. putida* GPo1 AlkB solubilised in LDAO, and a generic in vitro activity assay that allows for the direct testing of hydroxylation of the hydrophobic substrates. These new methods are likely to pave the way towards a better understanding of the structure and mechanism of action of the bacterial alkane hydroxylase system.

Acknowledgements The authors thank Prof. Nigel Scrutton and Prof. David Leys at Manchester Interdisciplinary Biocentre for providing recombinant *P. putida* rubredoxin reductase and *E. coli* TG1 cells transformed with the PKR10 plasmid. AR is an Australian Research Council Research Fellow. This work was supported by the Australia–India Strategic Research Fund of the Australian Government (grant BF040030).

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