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Catalytic residues and a predicted structure of tetrahydrobiopterin-dependent alkylglycerol mono-oxygenase

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Alkylglycerol mono-oxygenase (EC 1.14.16.5) forms a third, distinct, class among tetrahydrobiopterin-dependent enzymes in addition to aromatic amino acid hydroxylases and nitric oxide synthases. Its protein sequence contains the fatty acid hydroxylase motif, a signature indicative of a di-iron centre, which contains eight conserved histidine residues. Membrane enzymes containing this motif, including alkylglycerol mono-oxygenase, are especially labile and so far have not been purified to homogeneity in active form. To obtain a first insight into structure–function relationships of this enzyme, we performed site-directed mutagenesis of 26 selected amino acid residues and expressed wild-type and mutant proteins containing a C-terminal Myc tag together with fatty aldehyde dehydrogenase in Chinese-hamster ovary cells. Among all of the acidic residues within the eight-histidine motif, only mutation of Glu^{137} to alanine led to an 18-fold increase in the Michaelis–Menten constant for tetrahydrobiopterin, suggesting a role in tetrahydrobiopterin interaction. A ninth additional histidine residue essential for activity was also identified. Nine membrane domains were predicted by four programs: ESKW, TMHMM, MEMSAT and Phobius. Prediction of a part of the structure using the Rosetta membrane *ab initio* method led to a plausible suggestion for a structure of the catalytic site of alkylglycerol mono-oxygenase.

Key words: alkylglycerol mono-oxygenase, fatty acid hydroxylase motif, phenylalanine hydroxylase, tetrahydrobiopterin, transmembrane domain.

INTRODUCTION

Alkylglycerol mono-oxygenase (glycerylether mono-oxygenase, EC 1.14.16.5) is the only enzyme known to cleave the ether bond of alkylglycerols [1]. Alkylglycerols form an abundant and diverse class of lipids and phospholipids of humans and other animals and are thought to play a role in sperm and nerve development, in protection of the eye from cataract and in signal transduction of cytokines [2]. Alkylglycerol mono-oxygenase might be important for the regulation of ether lipid concentrations *in vivo*. Alkylglycerol mono-oxygenase is known to require tetrahydrobiopterin for catalysis [3]. The sequence of this enzyme remained obscure for decades owing to its lability that precluded its purification. Using bioinformatic database searches and recombinant expression of selected candidate genes, we recently found a predicted membrane protein with unknown function to encode alkylglycerol mono-oxygenase [4]. This sequence has no similarity to either of the other two classes of tetrahydrobiopterin-dependent enzymes, the aromatic amino acid hydroxylases and the nitric oxide synthases [5], and thus forms a third, distinct, class among tetrahydrobiopterin-dependent enzymes [4]. The detection of the fatty acid hydroxylase motif in this sequence gave a plausible explanation why purification attempts had failed so far. Proteins containing this motif are thought to contain a di-iron centre for catalytic activity, and none of the membrane proteins of this class which comprises hydroxylases and desaturases of specific lipid and steroid substrates has, so far, ever been isolated in active form [6].

Despite intense efforts, we also failed to isolate the enzyme in active form. We succeeded, however, in recombinant expression of a tagged version of the active protein in CHO (Chinese-hamster ovary) cells, thus opening up the possibility to investigate the contribution of specific amino acid residues to enzymatic activity of the protein by site-directed mutagenesis. With this tool, we had already shown the necessity of the eight conserved histidine residues for alkylglycerol mono-oxygenase activity [4], and we extend this analysis in the present study describing 26 further mutations, including a residue which, when mutated, attenuates tetrahydrobiopterin affinity to the enzyme 18-fold.

No protein of known structure in the PDB showed any similarity to alkylglycerol mono-oxygenase, precluding the prediction of a structure based on a known structure of a similar protein. Since purification and crystallization of this labile membrane protein appeared remote for experimental reasons, we predicted transmembrane domains and a structure of alkylglycerol mono-oxygenase using the Rosetta membrane *ab initio* method [7].

EXPERIMENTAL

Site-directed mutagenesis of selected residues in human alkylglycerol mono-oxygenase

Human alkylglycerol mono-oxygenase in pEXPR-IBA103 (IBA) harbouring a 6× Myc tag at the C-terminus [4] was used as template. Selected residues were altered to alanine using the QuikChange® site-directed mutagenesis kit (Stratagene). Primer sequences are listed in Supplementary Table S1 at http://www.BiochemJ.org/bj/443/bj4430279add.htm. Correct insertion of all mutations was confirmed by DNA sequencing.

Abbreviations used: CHO, Chinese-hamster ovary; GFP, green fluorescent protein.

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Transfection of CHO-K1 cells with wild-type and mutant alkylglycerol mono-oxygenase constructs

CHO-K1 (LGC Promochem) cells were plated in a six-well plate at a density of 3.75×10^5 cells in 3 ml of medium per well. After 24 h, two wells for each construct were transfected with 1 μg of human alkylglycerol mono-oxygenase–6×Myc plasmid DNA and 1 μg of human fatty aldehyde dehydrogenase (GenBank® accession number NM_000382) in the pcDNA3.1 + expression vector (Invitrogen) using ExGen 500 (Fermentas) following the manufacturer's protocol. Transfection efficiency was checked by GFP (green fluorescent protein) expression using N1-eGFP (Clontech Europe). Cells were cultivated at 37 °C under 5 % CO_2 in F-12K (Kaighn's modification of Ham's F12 medium) nutrient medium (Invitrogen), supplemented with 10 % (v/v) heat-inactivated fetal bovine serum (PAN Biotech) in the presence of 1 μM tetrahydrobiopterin precursor sepiapterin (Schircks Laboratories). After 48 h, cells were harvested by trypsinization. Cells transfected with wild-type enzyme and cells transfected with GFP (both co-transfected with human fatty aldehyde dehydrogenase) were prepared and analysed in parallel to serve as positive and negative controls respectively. For membrane preparation, cells were suspended in distilled water containing 1× protein inhibitor mixture (GE Healthcare), snap-frozen and stored at − 80 °C until analysis.

Alkylglycerol mono-oxygenase activity assay

Enzymatic activity was measured in supernatants of transfected cells harvested and shock frozen in 0.5 % CHAPS (Sigma). After thawing, mixing and centrifuging at 16000 ***g*** for 5 min at 4 °C, supernatants were incubated with 1-*O*-pyrenedecyl-*sn*-glycerol (chemically synthesized from pyrenedecanoic acid and glycerol as described in [8]), 0.2 mM tetrahydrobiopterin (Schircks Laboratories), 0.2 μg/ml (0.5 μmol/ml per min) recombinant dihydropteridine reductase from *Physarum polycephalum* [9], 0.1 mg/ml catalase (Sigma), 20 μmol·ml^{-1}·min^{-1} recombinant rat fatty aldehyde dehydrogenase [10], 0.2 mM NAD (Sigma) and 0.2 mM NADPH (Sigma) in 100 mM Tris/HCl (pH 8.5) for 60 min at 37 °C in a total volume of 10 μl. The reaction was started by the addition of protein and stopped by adding 30 μl of methanol. After centrifugation at 16000 ***g*** for 5 min, 10 μl of the resulting mixture was injected on to a Zorbax XDB-C_8 rapid resolution column (Agilent Technologies) using an Agilent 1200 Series HPLC system. Elution was performed at a flow rate of 1 ml/min starting with a mixture of 21 % (v/v) 10 mM potassium phosphate buffer (pH 6.0) and 79 % (v/v) methanol for 4.5 min followed by a gradient to 100 % methanol at 5.0 min. At 8.0 min, the 21:79 buffer/methanol mixture was re-established and the column was equilibrated for 30 s before starting the next run. Pyrenedecanoic acid was detected by fluorescence (340 nm excitation and 400 nm emission, detection limit 1 nM [8]). Fatty aldehyde dehydrogenase was provided both by transfection and in the assay mixture to ensure complete conversion of the intermediate product from the alkylglycerol mono-oxygenase reaction, the fluorescent aldehyde, into the detected compound pyrenedecanoic acid. Negative controls without protein (concentration of pyrenedecanoic acid <1 nM) and rat liver microsomes as positive controls were always run in parallel. For determination of K_m values, cell extracts were incubated with assay mixtures containing 0, 10, 30 or 100 μM tetrahydrobiopterin or 0, 1, 3, 10, 30 or 100 μM 1-*O*-pyrenedecyl-*sn*-glycerol.

The iron affinity of wild-type and E137A mutant alkylglycerol mono-oxygenase was tested using a phenanthroline inhibition assay. This method was chosen on the basis of previous results [11] showing no further increase in alkylglycerol mono-oxygenase activity upon addition of iron. Cell extracts were diluted 1:10 in increasing amounts (assay end concentration ranging from 33.75 nM to 3.375 mM) of the iron chelator phenanthroline in an optimized buffer containing 50 mM triethanolamine/HCl (pH 7.5), 150 mM NaCl, 10 % (v/v) glycerol, 10 mM ascorbic acid and 20 μM tetrahydrobiopterin. Mixtures were shaken at 25 °C for 15 min and were subsequently subjected to the standard activity assay, but in the presence of 0.4 mM tetrahydrobiopterin to saturate the E137A mutant with the cofactor. For at least three separate sets of experiments, curves were fitted to the data using GraphPad Prism 5.03, and the concentrations yielding half-maximal activity or 50 % inhibition were taken as K_m or IC_{50} respectively.

Phenylalanine hydroxylase activity assay and iron-binding studies

Recombinant rat liver phenylalanine hydroxylase was expressed as a maltose-binding protein-fusion protein, purified by affinity chromatography [11]. The E286A mutation was introduced using the QuikChange® site-directed mutagenesis kit, and the identity of the mutation was confirmed by DNA sequencing. Primer sequences are given in Supplementary Table S1. Phenylalanine hydroxylase proteins (0.5 mg/ml) were pre-incubated with increasing concentrations of phenanthroline in the presence of 1.2 mM phenylalanine, 1.2 mg/ml catalase [freed from low-molecular-mass compounds by gel filtration over NAP5 columns (GE Healthcare)] in 60 mM Hepes buffer (pH 7.0) for 15 min at 25 °C in 30 μl. The reaction was started by the addition of 6 μl of a solution containing 30 mM each of tetrahydrobiopterin and dithioerythritol to yield final concentrations of 5 mM tetrahydrobiopterin (to also saturate the E286A mutant with the cofactor), 1 mM phenylalanine, 1 mg/ml catalase and 5 mM dithioerythritol in 50 mM Hepes (pH 7.0). After incubation for 20 min at 37 °C, the reaction was stopped by the addition of 3.6 μl of 40 % (w/v) trichloroacetic acid. Tyrosine concentrations were measured after reversed-phase HPLC with fluorescence detection (275 nm excitation, 310 nm emission) as described in [11].

Preparation of membrane fractions and Western blotting

Membrane fractions of the cells were prepared as described in [4], protein concentration was determined using the Bradford assay with BSA as standard. To quantify the amount of recombinant tagged protein formed, Western blots were performed as described in [4]. In short, 40 μg of microsomal protein was separated by SDS/PAGE (10 % gels), transferred on to PVDF membranes and stained with rabbit polyclonal anti-c-Myc (A-14) antibody (1:1000 dilution; Santa Cruz Biotechnology) and horseradish-peroxidase-conjugated anti-(rabbit IgG) (1:10000 dilution; Pierce). Bands were visualized with the ECL (enhanced chemiluminescence) Plus detection system (GE Healthcare) and blots were scanned using a Typhoon 9410 scanner (457 nm excitation, 520 nm emission, 40 nm band pass, 500 V photomultiplier voltage; GE Healthcare). Wild-type and mutant alkylglycerol mono-oxygenase activities were normalized on Western blot band pixel count quantified with ImageQuant TL software (GE Healthcare).

Consensus sequence of alkylglycerol mono-oxygenase from 11 animal species

Alkylglycerol mono-oxygenase sequences from 11 animal species (*Rattus norvegicus*, *Mus musculus*, *Bos taurus*, *Equus caballus*, *Danio rerio*, *Pan troglodytes*, *Xenopus tropicalis*, *Macaca*

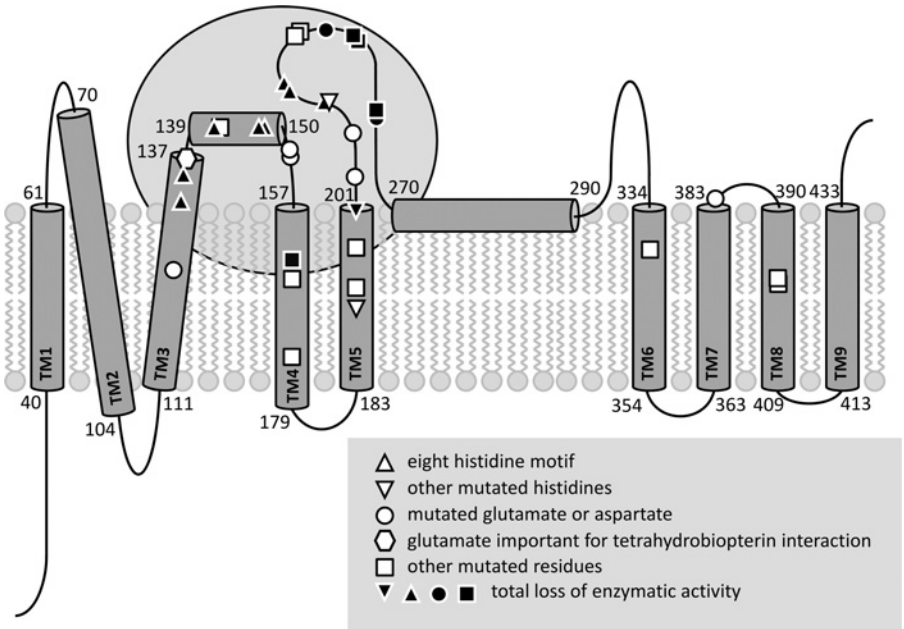

Figure 1 Transmembrane regions and residues mutated in alkylglycerol mono-oxygenase

Transmembrane (TM) regions 1–5 as well as the helix spanning residues 139–150 were taken from *ab initio* predicted structures (see Figure 5). Transmembrane regions 6–9 and the helix spanning residues 270–290 were assigned as a consensus from four programs (ESKW, TMHMM, MEMSAT and Phobius). The symbols indicate the position of amino acids residues mutated. Residues with white frames and black filling are those that led to a total loss of enzymatic activity of the recombinant protein expressed in CHO cells. Upward-pointing triangles indicate the eight-histidine motif [4,25], downward-pointing triangles indicate further histidine residues that were mutated. The hexagon shows the position of Glu^{137} which is important for tetrahydrobiopterin interaction. Circles show positions of acidic residues (aspartate and glutamate) mutated, squares indicate other mutated residues. The region of the active site and the presumed di-iron centre is highlighted in grey.

mulatta, *Gallus gallus* and *Strongylocentrotus purpuratus* and *Homo sapiens*) were aligned by the ClustalW module of Mega 4.0 [12], using the identity option and all other parameters as default values. Sequences were taken from the protein database of the National Center for Biotechnology Information (NCBI).

Assignment of transmembrane regions

Transmembrane predictions are a consensus from four programs: ESKW [13], TMHMM [14], MEMSAT [15] and Phobius [16] for alkylglycerol mono-oxygenase sequences available from animal species, showing a consistent picture of nine conserved transmembrane regions predicted.

Rosetta *ab initio* prediction of an alkylglycerol mono-oxygenase structure

The Rosetta *ab initio* method is based on the assumption that the native state of a protein is at the global free energy minimum and uses structural information for short protein fragments (extracted from the PDB) to sample local conformations adopted by these fragments [17–19]. Specifically, structural fragment libraries for each three- and nine-residue segment of protein sequence of interest are extracted from the PDB using a sequence profile comparison method that uses multiple sequence alignment of the homologous segments. The Rosetta membrane environment-specific energy function favours burial of small hydrophobic residues and exposure of large hydrophobic residues within the hydrophobic layer of the membrane together with minimizing hydrophobic residue exposure in the polar environment outside the membrane [7]. The membrane environment is simulated using an energy function derived from statistics generated from known high-resolution membrane protein structures [7]. This membrane environment-specific energy function includes residue environment, residue–residue interactions and residue density terms. The residue environment term is based on amino acid propensities to be in distinct membrane layers (hydrophobic, interface, polar and water) and depends on the residue burial state, from being completely buried within a protein environment to being completely exposed either to the lipid or water environments. Residue–residue interaction terms are based on the propensities of amino acid pairs to be in close proximity to each other within hydrophobic and polar layers. The residue density term is based on the distribution of the number of residue neighbours within 6 and 12 Å (1 Å = 0.1 nm) spheres in native membrane protein structures. The membrane environment-specific full atom energy function is similar to the energy function used for modelling of water-soluble proteins [20], except that solvation and hydrogen-bond potentials are functions of residue depth in the membrane. The membrane environment is described by two isotropic phases (water and hydrocarbon layers) and an anisotropic phase (interface layer) that interpolates the chemical properties of the two isotropic phases. An implicit atomic solvation potential for the hydrophobic layer is based on experimental transfer free energies of peptides from water to lipid bilayers [21]. The atomic solvation energies in the water environment are based on a solvation model developed by Lazaridis and Karplus [22]. The atomic solvation energies in the membrane interface layer were derived by interpolating the solvation properties between hydrophobic and water layers using sigmoidal function. We applied the Rosetta membrane *ab initio* method to modelling of human alkylglycerol mono-oxygenase. Transmembrane helix topology of human alkylglycerol mono-oxygenase was predicted using the methods described above, and all five predicted transmembrane regions for the Pro^{37}–Ile^{205} region were required to span the membrane during modelling. A total of 5000 low-resolution *de novo* models of human alkylglycerol mono-oxygenase were generated using

the Rosetta membrane *ab initio* method followed by model clustering as described previously [23]. Centre models from each of the five largest clusters were subjected to all-atom refinement protocol [24], and the best model was chosen on the basis of available experimental data. Expansion of the prediction to fully cover the eight-histidine motif [25] did not yield consistent models, as inclusion of further transmembrane regions inflated dimensionality of structure prediction. The co-ordinates of the predicted structure for residues Pro[37]–Ile[205] are available in PDB format at http://www.BiochemJ.org/bj/443/bj4430279add.htm.

Presentation of data and statistical analysis

Results are presented as means ± S.E.M. for the indicated number of independent experiments. Statistical analysis using Student's or one sample *t* test was performed using GraphPad Prism 5.03. *P* values <0.05 were considered significant.

RESULTS

Loss-of-function mutants

We have shown previously that all eight histidine residues representing the fatty acid hydroxylase motif are essential for the enzymatic activity of alkylglycerol mono-oxygenase [4]. We now expanded this investigation to another 26 conserved residues ranging from position 125 to position 397 of the human protein sequence (Figure 1). In addition to the eight histidine residues, we identified seven further residues (Gln[162], His[201], Asp[233], Asn[235], Tyr[236], Trp[243] and Asp[244]) which caused a total loss of activity when mutated to alanine (Figures 1 and 2). Depending on the amount of protein expressed, the detection limit was 1–5 % of wild-type activity. Three of these amino acids (His[201], Trp[243] and Asp[244]) are conserved among all animal members of the fatty acid hydroxylase family [4].

Mutants with residual enzyme activity

In six of the mutants (Q146A, D153A, H220A, Y230A, C231A and C397A), activity was not significantly different from controls. None of the tested mutations yielded activity levels higher than that of wild-type (Figure 2). Twelve mutants had significantly reduced, but clearly detectable, enzyme activity (E137A, E152A, Q166A, Y174A, H189A, Q191A, Q197A, E203A, E212A, Y338A, D384A and R396A) (Figure 2). Two of these mutants (Q197A and Y338A) had an activity of less than 5 % of that of controls, which was nevertheless significantly higher than control values owing to good protein expression of the mutants (Figure 2).

Mutants with decreased protein expression

Eight of the mutant plasmids yielded significantly lower amounts of protein, five of them expressed less than half of the protein amount compared with wild-type (D125A, N235A, Y236A, W243A and D244A). In D125A, the amount of protein expressed was too low for a reliable quantification, and therefore this mutant was excluded from activity analysis (Figure 2).

Investigation of selected mutants for tetrahydrobiopterin interaction

Within the region of the conserved essential eight-histidine motif [25] which comprises residues 132–225 of human alkylglycerol mono-oxygenase, the catalytic core of the enzyme, we then selected mutants of all glutamate and aspartate residues (E137A, E152A, D153A, E203A and E212A) and

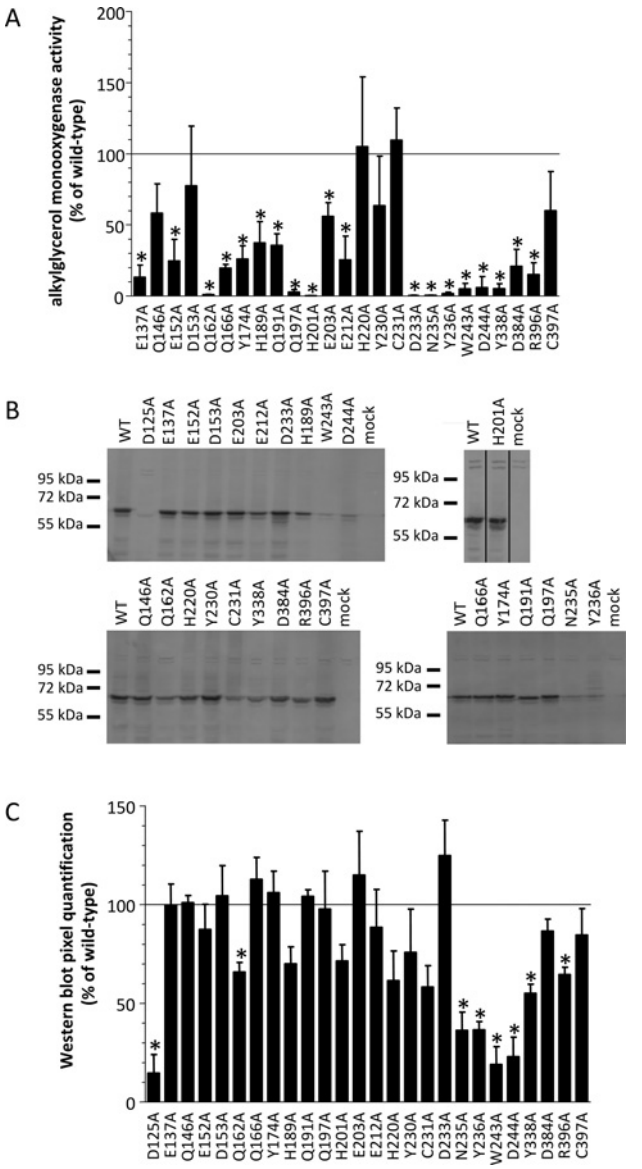

Figure 2 Enzymatic activities of 25 mutant alkylglycerol mono-oxygenase proteins expressed in CHO cells

(**A**) Alkylglycerol mono-oxygenase activity of individual experiments for 25 mutations were normalized to band pixels from Western blots and are shown in relation to wild-type activity measured in parallel experiments. One mutant (D125A) did not express detectable protein and was excluded from activity evaluation. Asterisks indicate significant differences from 100 % wild-type level ($P < 0.05$). Results are means ± S.E.M. for at least three experiments. (**B**) Representative Western blots of membrane fractions of all 26 mutant alkylglycerol mono-oxygenase proteins after heterologous transfection in CHO cells. Mock indicates cells transfected with human fatty aldehyde dehydrogenase and GFP. Protein marker bands are indicated on the left-hand side of each blot (with sizes given in kDa). For the blot of H201A, wild-type (WT) and mock lanes were separated from the mutant on the original blot by other samples which were cut out for clarity as indicated by the lines in the Figure. (**C**) Expression of recombinant proteins quantified from scans from at least three experiments. Asterisks indicate significant differences from 100 % wild-type level ($P < 0.05$).

an additional histidine mutant (H189A), which all had detectable residual activity. We tested these mutants for their concentration-dependence of alkylglycerol mono-oxygenase activity with respect to tetrahydrobiopterin and 1-*O*-pyrenedecyl-*sn*-glycerol. Cell extracts expressing wild-type enzyme were chosen as controls. Although no significant changes could be detected in the affinity of mutants towards the substrate (Figure 3A), mutant E137A had an 18.3-fold increased

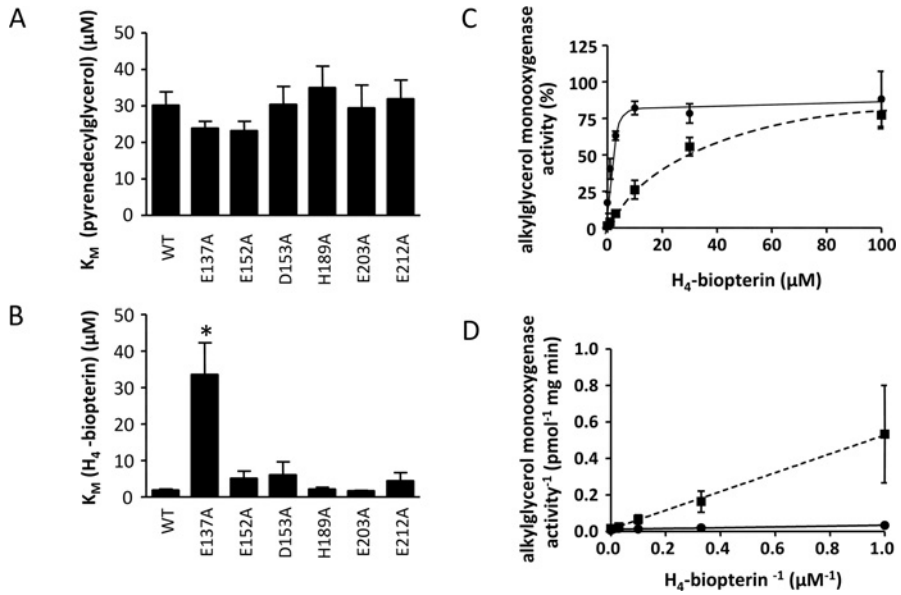

Figure 3 Michaelis–Menten constants of six selected mutant proteins for tetrahydrobiopterin and 1-*O*-pyrenedecyl-*sn*-glycerol

The selected residues include all glutamate and aspartate residues within the conserved eight-histidine motif. CHO cells transfected with wild-type and mutant alkylglycerol mono-oxygenase were incubated with increasing amounts of tetrahydrobiopterin (H_4-biopterin) or 1-*O*-pyrenedecyl-*sn*-glycerol. Activities were quantified by analysis of pyrenedecanoic acid with HPLC. (**A**) Michaelis–Menten constants for the substrate 1-*O*-pyrenedecyl-*sn*-glycerol ($n = 3$). (**B**) Michaelis–Menten constants for tetrahydrobiopterin (H_4-biopterin) from three independent experiments. $^*P < 0.05$, relative to wild-type constants. (**C**) Tetrahydrobiopterin dependence of wild-type (continuous line, ●) and E137A (broken line, ■), linear representation. (**D**) Double-reciprocal plot of the data shown in (**C**).

K_m value for tetrahydrobiopterin in comparison with wild-type ($33.5 \pm 8.79\ \mu$M compared with $1.83 \pm 0.38\ \mu$M; $n = 3$; $P < 0.05$; Figure 3B). Figures 3(C) and 3(D) show the original data for tetrahydrobiopterin-dependence of the activity in E137A mutant and wild-type enzymes, in normal and in double reciprocal plotting respectively.

Iron affinity of wild-type and E137A alkylglycerol mono-oxygenase

To check whether mutant E137A also displayed a different affinity not only for tetrahydrobiopterin, but also for iron, which is essential for catalysis [11], we incubated E137A- and wild-type-transfected CHO cell extracts with increasing amounts of the iron chelator phenanthroline and subsequently assayed their residual alkylglycerol-cleaving ability. Activity of both wild-type alkylglycerol mono-oxygenase and the E137A mutant alkylglycerol mono-oxygenase were competitively inhibited by phenanthroline (Figure 4A). IC_{50} values for mutant E137A ($0.69 \pm 0.19\ \mu$M; $n = 5$) were 5-fold lower than values for wild-type ($3.41 \pm 0.64\ \mu$M; $n = 5$; $P < 0.01$). Thus the E137A mutant of alkylglycerol mono-oxygenase had an approximately 5-fold lower affinity for iron.

Iron affinity of wild-type and E286A phenylalanine hydroxylase

Since iron affinity of the phenylalanine hydroxylase E286A mutant which also displays a highly increased K_m value for tetrahydrobiopterin ([26], and see the Discussion) had not been investigated so far, we investigated iron binding of this mutant in comparison with the wild-type enzyme with a similar phenanthroline competition activity assay using recombinant *Escherichia coli*-expressed enzymes for comparative purposes (Figure 4B). Both wild-type and mutant phenylalanine hydroxylase were inhibited by phenanthroline. The corresponding IC_{50} values for phenanthroline were $6.37 \pm 0.23\ \mu$M for the E286A mutant phenylalanine hydroxylase and $15.7 \pm 1.51\ \mu$M for the wild-type phenylalanine hydroxylase ($n = 3$ each; $P < 0.01$). Thus the E286A mutation in phenylalanine hydroxylase led to an approximately 2.5-fold decrease in iron affinity.

Ab initio predicted structure of alkylglycerol mono-oxygenase

Figure 5 shows the structure of residues 37–205 modelled by Rosetta Membrane. Even though no information on residues critical for protein function were used for model generation, all residues leading to a total loss of enzyme activity upon alanine mutation are found in close spatial proximity. Moreover, all five histidine residues of the eight-histidine motif covered in the modelled region, as well as the highly conserved His^{201}, form a shallow cavity ready to accommodate a catalytically active iron cluster that could not be included in the actual model. Glu^{137}, the residue involved in the interaction with tetrahydrobiopterin, is found in a flexible loop region close to the histidine motif at the surface of the predicted partial structure accessible for the cofactor.

DISCUSSION

The present paper is a first report on structure–function relationships of alkylglycerol mono-oxygenase, the only enzyme known to cleave the ether bond of alkylglycerols. Owing to the instability inherent to the membrane proteins containing the fatty acid hydroxylase motif, neither alkylglycerol mono-oxygenase nor a similar protein of this family has ever been purified, and therefore also no structures of similar proteins are available in databases. Even the sequence of alkylglycerol mono-oxygenase has only been assigned recently by recombinant expression of bioinformatically selected candidate genes [4]. In the present study, we investigated the impact of mutation of conserved residues on alkylglycerol mono-oxygenase activity, identified a residue important for tetrahydrobiopterin interaction and present an *ab initio* predicted structure of a part of the enzyme.

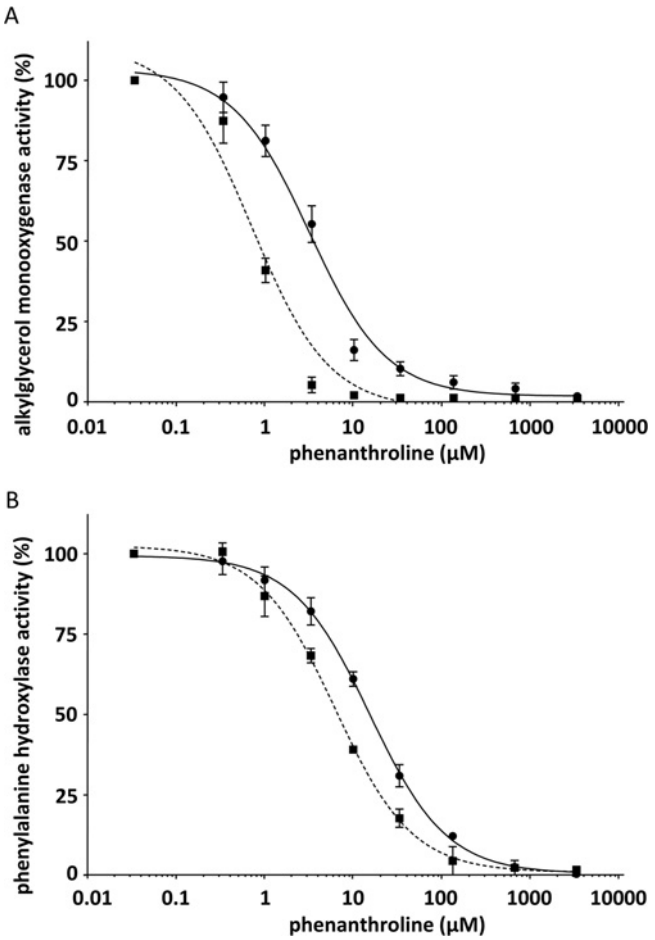

Figure 4 Influence of tetrahydrobiopterin-affinity-lowering glutamate to alanine mutations on iron affinity in alkylglycerol mono-oxygenase and phenylalanine hydroxylase

(**A**) Dependence of enzymatic activity of wild-type and E137A alkylglycerol mono-oxygenase on concentrations of the iron chelator phenanthroline. Extracts of CHO cells transfected with wild-type (continuous line, ●) and E137A mutant (broken line, ■) alkylglycerol mono-oxygenase were incubated for 15 min at 25 °C with increasing amounts of phenanthroline. Alkylglycerol mono-oxygenase activities were quantified by analysis of pyrenedecanoic acid formation by HPLC with fluorescence detection in presence of 0.4 mM tetrahydrobiopterin. Activity of the lowest phenanthroline concentration was set to 100 %. Under standard assay conditions, the E137A mutant shows 12.8 ± 4.8 % of enzymatic activity compared with wild-type alkylglycerol mono-oxygenase (Figure 2). Results are means ± S.E.M. ($n = 5$). (**B**) Dependence of enzymatic activity of wild-type and E286A mutant phenylalanine hydroxylase on concentrations of phenanthroline. Recombinant wild-type (continuous line, ●) and E286A mutant (broken line, ■) rat phenylalanine hydroxylase were incubated for 15 min at 25 °C with increasing amounts of phenanthroline. Phenylalanine hydroxylase activity was measured by monitoring the formation of tyrosine by HPLC with fluorescence detection in the presence of 5 mM tetrahydrobiopterin. Activity of the lowest phenanthroline concentration was set to 100 %. Under standard assay conditions with 5 mM tetrahydrobiopterin, the E286A mutant showed 7.9 ± 1.4 % of enzymatic activity compared with wild-type phenylalanine hydroxylase. Results are means ± S.E.M. ($n = 3$).

Earlier work showed that eight conserved histidine residues are essential for the function of fatty acid hydroxylase motif-containing enzymes [25], and these eight are required for alkylglycerol mono-oxygenase activity [4]. However, when analysing the protein sequences, three further residues were found to be conserved among all investigated fatty acid hydroxylase motif-containing animal proteins, including alkylglycerol mono-oxygenase. These residues are His^{201}, Trp^{243} and Asp^{244} [4]. We found that mutation of any of these residues also abolished activity of alkylglycerol mono-oxygenase, suggesting that, in addition to the eight histidine residues, these residues might be necessary for fatty acid hydroxylase function in general. The two further histidine residues conserved in all animal alkylglycerol mono-oxygenase sequences, His^{189} and His^{220}, however, were not essential for activity. All residues essential for enzymatic activity cluster to a region which we consider to be the catalytic core of the enzyme (see Figure 1).

In previous work, we have demonstrated that tetrahydrobiopterin interaction and metal-dependence of alkylglycerol mono-oxygenase is similar to tetrahydrobiopterin-dependent aromatic amino acid hydroxylases, but different from nitric oxide synthases [11]. In aromatic amino acid hydroxylases, a conserved glutamate residue (Glu^{286} in human phenylalanine hydroxylase) interacts with tetrahydrobiopterin in the crystal structure [27]. Mutation of this glutamate residue to alanine leads to a highly increased K_m value for tetrahydrobiopterin [26]. In the present study, we mutated all glutamate and aspartate residues within the catalytic site of alkylglycerol mono-oxygenase to alanine and assessed the K_m values of the mutants when expressed in CHO cells. Only one of these mutants, E137A, had an 18-fold increased K_m value for tetrahydrobiopterin, whereas the K_m values of all other mutants selected for tetrahydrobiopterin were similar to that of wild-type enzyme. Although E137A had a similar affinity for the alkylglycerol substrate as that of the wild-type enzyme, we observed a 5-fold weaker binding of iron by this mutant. This was less pronounced than the 18-fold effect of the mutation on the K_m value for tetrahydrobiopterin. Therefore the increased K_m value for tetrahydrobiopterin in E137A is unlikely to be a secondary effect caused by the decrease in iron affinity only. The effect of the tetrahydrobiopterin-affinity-lowering mutation E286A in phenylalanine hydroxylase on iron affinity had not been investigated previously. We therefore applied a similar phenanthroline competition assay to wild-type and E286A mutant recombinant rat phenylalanine hydroxylase for comparative purposes. We also found that, in phenylalanine hydroxylase, mutation of the tetrahydrobiopterin-binding Glu^{286} to alanine reduced iron affinity, although to a lesser extent (2.5-fold). The IC_{50} values for phenanthroline we observed in the present study for wild-type recombinant rat phenylalanine hydroxylase agree well with previous results of phenanthroline inhibition of native phenylalanine hydroxylase purified from rat liver [28].

It might be no coincidence that in both enzymes, alkylglycerol mono-oxygenase and phenylalanine hydroxylase, both tetrahydrobiopterin-interacting glutamate residues are adjacent to an essential histidine residue, followed by a spacer sequence of exclusively neutral amino acids and another essential histidine residue. For all tetrahydrobiopterin-dependent aromatic amino acid hydroxylases, the sequence environment of Glu^{286} is H-[DE]-[FILV]-[FILMVY]-G-H. This can be deduced from the core of the PDOC00316 PROSITE motif [29] which recognizes all known enzymes of this type. For alkylglycerol mono-oxygenase, in 11 animal species the consensus sequence around Glu^{137} is H-E-[ILV]-N-I-[FILM]-W-A-[AG]-H. In aromatic amino acid hydroxylases, both of these conserved histidine residues bind the catalytically active non-haem iron [27]. Alkylglycerol mono-oxygenase also requires a non-haem iron [11], and the two conserved histidine residues adjacent to Glu^{137} are therefore good candidates for iron interaction. Fatty acid hydroxylase motif-containing proteins, however, use a di-iron centre for catalysis [6]. Thus iron chelation and biochemistry might be more complex in alkylglycerol mono-oxygenase than in phenylalanine hydroxylase. No protein containing a fatty acid hydroxylase motif other than alkylglycerol mono-oxygenase has ever been reported to require tetrahydrobiopterin for catalysis. Intriguingly, none of these enzymes except alkylglycerol mono-oxygenase has a

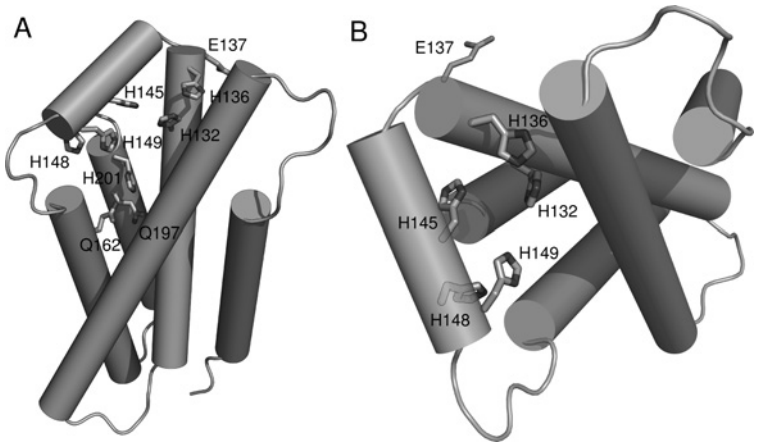

Figure 5 Rosetta *ab initio* predicted partial structure of alkylglycerol mono-oxygenase

Three-dimensional structure of alkylglycerol mono-oxygenase residues Pro^{37}–Ile^{205} including five transmembrane regions predicted using the Rosetta membrane method [7] visualized using PyMOL (http://www.pymol.org). (**A**) Helical regions are shown in tube representation, and residues leading to inactivation of alkylglycerol mono-oxygenase upon mutation to alanine and Glu^{137} crucial for tetrahydrobiopterin binding are highlighted as sticks. (**B**) Close-up of the suspected active site of alkylglycerol mono-oxygenase. All five modelled histidine residues of the eight-histidine motif as well as Glu^{137} are found in close spatial proximity forming a shallow cavity ready to accommodate an iron cluster.

glutamate or aspartate residue adjacent to the conserved histidine residue at this position in its protein sequence (see Supplementary Figure 7 in [4]).

Ab intio structure prediction using Rosetta membrane method yielded a plausible three-dimensional model for residues 37–205 of alkylglycerol mono-oxygenase. The structure presented (Figure 5) provides a structural explanation for all loss of activity mutations covered in the sequence, although this information was not used for model generation. Three-dimensional positioning of five histidine residues in close spatial proximity defines the localization of the catalytically active iron cluster not included in the actual model. The flexible Glu^{137} provides a binding partner for tetrahydrobiopterin next to the iron cluster, completing the redox system of alkylglycerol mono-oxygenase. A putative binding site for substrate ether lipids can be inferred from several apolar residues near the active site. The consensus of ESKW, TMHMM, MEMSAT and Phobius estimate the fifth transmembrane helix to range from amino acid residues 194 to 214. In the *ab initio* structure, however, this helix ranges from 183 to 201. The *ab initio* structure is more plausible in this point, because His^{201} in the *ab initio* structure is not buried in the membrane, but becomes accessible to the catalytic region of the enzyme. As discussed above, His^{201} is conserved among all fatty acid hydroxylase motif-containing proteins and is essential for alkylglycerol mono-oxygenase activity.

AUTHOR CONTRIBUTION

Katrin Watschinger, Julian Fuchs and Vladimir Yarov-Yarovoy performed experiments and calculations. Markus Keller and Albin Hermetter contributed new reagents and analytical tools. Nicolas Hulo and Ernst Werner performed sequence analyses and selected residues for mutation. Katrin Watschinger, Julian Fuchs, Vladimir Yarov-Yarovoy, Georg Golderer and Gabriele Werner-Felmayer analysed data. Katrin Watschinger, Julian Fuchs, Vladimir Yarov-Yarovoy and Ernst Werner wrote the paper.

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online data

SUPPLEMENTARY ONLINE DATA

Catalytic residues and a predicted structure of tetrahydrobiopterin-dependent alkylglycerol mono-oxygenase

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Table S1 Forward and reverse primer sequences for introduction of mutations by QuikChange® site-directed mutagenesis

(a) Alkylglycerol mono-oxygenase

Mutation	Forward primer (5′→3′)	Reverse primer (5′→3′)
D125A	GCCTTCTTAGGAGTTGCCTTTGGCTACTACTGG	CCAGTAGTAGCCAAAGGCAACTCCTAAGAAGGC
E137A	CCATCGTATGGCGCATGCAGTTAATATTATGTGGGCC	GGCCCACATAATATTAACTGCATGCGCCATACGATGG
Q146A	ATGTGGGCCGGGCATGCAACACATCATAGTTCTGAAGAC	GTCTTCAGAACTATGATGTGTTGCATGCCCGGCCCACAT
E152A	CACATCATAGTTCTGCAGACTATAACTTATCCACAGC	GCTGTGGATAAGTTATAGTCTGCAGAACTATGATGTG
D153A	CACATCATAGTTCTGAAGCTTATAACTTATCCACAGCACTGAG	CTCAGTGCTGTGGATAAGTTATAAGCTTCAGAACTATGATGTG
Q162A	CTTATCCACAGCACTGCGCGCGTCTGTCCTCCAGATAT	ATATCTGGAGGACAGACGCGCGCAGTGCTGTGGATAAG
Q166A	CACTGAGACAGTCTGTCCTCGCGATATATACTTCCTGG	CCAGGAAGTATATATCGCGAGGACAGACTGTCTCAGTG
Y174A	CTTCCTGGATTTTCGCGAGTCCCCTGGCCCTCTTC	GAAGAGGGCCAGGGGACTCGCGAAAATCCAGGAAG
H189A	CCCCCTTCAGTATATGCTGTAGCGCTTCAATTCAATCTTCTTTACC	GGTAAAGAAGATTGAATTGAAGCGCTACAGCATATACTGAAGGGGG
Q191A	CCCCTTCAGTATATGCTGTTCATCTTGCATTCAATCTTCTTTACC	GGTAAAGAAGATTGAATGCAAGATGAACAGCATATACTGAAGGGG
Q197A	CTTCAATTCAATCTTCTTTACGCGTTTTGGATCCATACAGAGGTC	GACCTCTGTATGGATCCAAAACGCGTAAAGAAGATTGAATTGAAG
H201A	CTTTACCAATTTTGGATCGCTACAGAGGTCATCAATAACC	GGTTATTGATGACCTCTGTAGCGATCCAAAATTGGTAAAG
E203A	GGATCCATACAGCTGTCATCAATAACCTTGGTCC	GGACCAAGGTTATTGATGACAGCTGTATGGATCC
E212A	CCTTGGTCCTTTAGCGCTGATTCTTAATACTCCTAGCC	GGCTAGGAGTATTAAGAATCAGCGCTAAAGGACCAAGG
H220A	GATTCTTAATACTCCTAGCGCTCATAGGGTTCATCATGGCAG	CTGCCATGATGAACCCTATGAGCGCTAGGAGTATTAAGAATC
Y230A	CATGGCAGAAATCGTGCATGCATAGACAAAAATTATGCTGGTG	CACCAGCATAATTTTTGTCTATGCATGCACGATTTCTGCCATG
C231A	CATCATGGCAGAAATCGGTACGCCATAGACAAAAATTATGCTGG	CCAGCATAATTTTTGTCTATGGCGTACCGATTTCTGCCATGATG
D233A	CATGGCAGAAATCGTTATTGCATCGCGAAAAATTATGCTGGTGTTC	GAACACCAGCATAATTTTTCGCGATGCAATAACGATTTCTGCCATG
N235A	GGCAGAAATCGTTATTGCATAGACAAAGCTTATGCTGGTGTTC	GAACACCAGCATAAGCTTTGTCTATGCAATAACGATTTCTGCC
Y236A	CGTTATTGCATAGACAAAAATGCGGCCGGTGTTCTTATTATTTGGG	CCCAAATAATAAGAACACCGGCCGCATTTTTGTCTATGCAATAACG
Y338A	CATCTCAGCTATTAAAGATAGCTACAGTTGTACAGTTTGCTCTG	CAGAGCAAACTGTACAACTGTAGCTATCTTTAATAGCTGAGATG
W243A	GCTGGTGTTCTTATTATTGCGGATAAGATTTTTGGGACATTTGAAGC	GCTTCAAATGTCCCAAAAATCTTATCCGCAATAATAAGAACACCAGC
D244A	GCTGGTGTTCTTATTATTTGGGCTAAGATTTTTGGGACATTTGAAGC	GCTTCAAATGTCCCAAAAATCTTAGCCCAAATAATAAGAACACCAGC
D384A	GACTTCCATTGGATTTCTGCTAGCTCAAAGACCCAAGGCAGC	GCTGCCTTGGGTCTTTGAGCTAGCAGAAATCCAATGGAAGTC
R396A	GGCAGCTATTATGGAAACTCTCGCATGCTTGATGTTCTTAATGC	GCATTAAGAACATCAAGCATGCGAGAGTTTCCATAATAGCTGCC
C397A	GGAAACTCTCCGAGCGCTGATGTTCTTAATGCTGTACCG	CGGTACAGCATTAAGAACATCAGCGCTCGGAGAGTTTCC

(b) Phenylalanine hydroxylase

Mutation	Forward primer (5′→3′)	Reverse primer (5′→3′)
E286A	CCTGAACCTGACATCTGCCACGCGTTGTTGGGACATGTGCC	GGCACATGTCCCAACAACGCGTGGCAGATGTCAGGTTCAGG

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