Evidence linking the *Pseudomonas oleovorans* alkane ω-hydroxylase, an integral membrane diiron enzyme, and the fatty acid desaturase family

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Abstract *Pseudomonas oleovorans* alkane ω-hydroxylase (AlkB) is an integral membrane diiron enzyme that shares a requirement for iron and oxygen for activity in a manner similar to that of the non-heme integral membrane desaturases, epoxidases, acetyltransferases, conjugases, ketolases, dehydrogenases, and methyl oxidases. No overall sequence similarity is detected between AlkB and desaturase-like enzymes by computer algorithms; however, they do contain a series of histidine residues in a similar relative positioning with respect to hydrophobic regions thought to be transmembrane domains. To test whether these conserved histidine residues are functionally equivalent to those of the desaturase-like enzymes we used scanning alanine mutagenesis to test if they are essential for activity of AlkB. These experiments show that alanine substitution of any of the eight conserved histidines results in complete inactivation, whereas replacement of three non-conserved histidines in close proximity to the conserved residues, results in only partial inactivation. These data provide the first experimental support for the hypotheses: (i) that the histidine motif in AlkB is equivalent to that in the desaturase-like enzymes and (ii) that the conserved histidine residues play a vital role such as coordinating the Fe ions comprising the diiron active site.

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1. Introduction

The alkane ω-hydroxylase (AlkB) system from *Pseudomonas oleovorans* is responsible for the initial oxidation of inactivated alkanes [1]. It is a three-component system comprising: (i) a soluble NADH-reducedoxin reductase [2], (ii) a soluble rubredoxin [3], and (iii) the integral membrane oxygenase AlkB (EC 1.14.15.3) [4–6]. In addition to the energetically demanding hydroxylation of inactivated methyl groups, AlkB has been shown to catalyze other reactions such as epoxidation of alkenes [7,8]. Reactions of inactivated hydrocarbons are reminiscent of transformations involving high-valent catalytic intermediates mediated by cytochrome P450 and methane monoxygenase [9–11].

Witholt’s group showed that AlkB can be overexpressed in *Escherichia coli* to levels comparable to those found in *Pseudomonas*, and that the protein has varying degrees of activity either in different *E. coli* hosts, or when grown under different conditions [12–14]. AlkB from *E. coli* forms lipoprotein vesicles with properties similar to those found in a natural source protein [15]. A high level of protein expression was also achieved when AlkB was placed under the control of T7 polymerase in *E. coli* [16]. The protein was purified to homogeneity and single turnover reactions, monitored by Mössbauer spectroscopy, revealed an exchange-coupled dinuclear iron center that participates in catalysis [16].

Several distinct classes of soluble diiron proteins have been identified that catalyze a diversity of oxygen-mediated chemistry on a variety of substrates [17]. Class I contains the soluble O2-carrying proteins hemerythrin and myohemerythrin, coordinated primarily via nitrogen ligands [18]. Class II includes the soluble methane monoxygenase, ribonucleotide reductase and soluble plant Δ^9-desaturases in which the diiron center is primarily coordinated via oxygen ligands [11,19–21].

In all diiron centers described to date, there is at least one bridging carboxylate ligand, raising the possibility that a similar carboxylate bridge may be involved in the diiron centers of integral membrane histidine motif-containing enzymes. Biochemical similarities between the soluble and integral membrane Δ^9-desaturases, along with an absence of motifs characteristic of Class I and II diiron centers, led to the hypothesis that the membrane desaturases contain a third class of diiron active site [19,22]. Mössbauer analysis of AlkB in the reduced state confirmed this hypothesis [16].

Sequence alignment of highly divergent integral membrane desaturases revealed a conserved tripartite motif consisting of eight histidines [22]. Inclusion of the Δ^9-desaturases from *Anaerobacter* [23] and *borage* [24] revealed that glutamine, another nitrogen-containing residue, can apparently substitute for the sixth histidine [25]. To reflect this, the motif can best be described as: H X/C0 Ê/H X/C0 Ô/H X/C0 Ô/H (H/Q) X/C0 Ô/H, but for the purposes of this paper it will be referred to as the eight-histidine motif. The same motif has now been identified in a number of other enzymes including desaturases, epoxidases, acetyltransferases, conjugases, ketolases, dehydrogenases, and methyl oxidases in addition to hydroxylases [25–27]. In all cases the three elements of the motif are conserved both in their order of occurrence and in their positions with respect to membrane spanning domains, such that all the histidine residues are predicted to lie in hydrophilic domains on the cytoplasmic face and are therefore potentially available to interact with each other [22,28,29].

Abbreviations: AlkB, alkane ω-hydroxylase; PCR, polymerase chain reaction

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The eight-histidine motif was shown to be important for the coenzyme A-dependent ω-desaturase because individual conversion of any of the eight conserved histidines into alanines eliminated its ability to complement a yeast strain deficient in ω-desaturase activity [22]. The importance of these histidine residues was subsequently confirmed for fatty acid desaturases that act on acyl lipids [30] and sterols [27].

We have previously identified the eight-histidine motif in AlkB, however, there is no detectable sequence similarity between AlkB and the desaturase family of enzymes outside of the occurrence of these eight histidine residues. Thus, we investigated the consequences of mutations in each of the residues of the motif along with several adjacent histidine residues for control purposes. Here we report the essential nature of the eight histidine residues that comprise the conserved motif for AlkB. These data provide the first experimental data to support a functional commonality between the distantly related AlkB and the previously characterized oxygen-, and iron-requiring enzymes that share the histidine motif and act on similarly inactivated substrates.

2. Materials and methods

2.1. Gene constructs

The wild-type alkB gene from *P. oleovorans* (GenBank J04618, [6]) in the *E. coli* expression plasmid pAlkB-3a [16] was used as the starting clone for these studies. Several restriction sites were introduced into the original coding sequence; uppercase characters are the accession characters are the coding characters are the non-coding. Residues of AlkB that were converted to alanines are indicated by stars. Conservative, or similar residues are indicated by black reverse type. The wild-type AlkB described above, was expressed in *E. coli* BL21(DE3) pLuxS cells under the control of the T7 polymerase as previously described [16]. Manipulations of cells and isolation of AlkB were carried out at 0–4°C whenever possible. A membrane fraction, highly enriched for AlkB, was isolated for activity measurements. This was accomplished by resuspension of the cells in a ratio of 1 g of packed cells/3.3 ml of 50 mM HEPES, pH 8.0 containing 4 mM MgCl₂, 2 mM phenylmethylsulfonyl fluoride, and, 3000 Kunitz units DNase I, and 10000 units of catalase per g of cells. The cell suspension was disrupted by passage through a French pressure cell with a 70 MPa pressure drop. The resulting extract was clarified by centrifugation at 30000 × g for 30 min. An AlkB-enriched membrane fraction was collected by centrifugation at 240000 × g for 1 h. The supernatant was decanted and the pelleted material resuspended in a minimal volume of 25 mM HEPES, pH 7.5 containing 10% (v/v) ethylene glycol by sonication. Aliquots of the membrane-enriched fraction were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) on an 12% gel. The gel was stained with Coomassie brilliant blue and the abundance of AlkB in each sample estimated by densitometry with the use of bovine serum albumin as a standard. The activity of AlkB was monitored spectrophotometrically as the octane-reductase as previously described [4]. One unit of activity (U) is defined as the oxidation of 1 μmol of NADPH per min at 22°C.

3. Results

AlkB is homologous to the xylene monooxygenase, XylM from *Pseudomonas putida* [33]. However, neither AlkB nor XylM shows significant sequence similarity to fatty acid desaturases and related enzymes when compared by standard computer algorithms such as BLAST. However, manual inspection revealed a series of histidine residues that have counterparts to an eight-histidine motif identified in fatty acid desaturases and related enzymes.

![Fig. 1. Comparison of regions Ia, Ib and II of the sequences containing the eight-histidine motif. The eight conserved histidines are denoted by black reverse type. Residues of AlkB that were converted to alanines are indicated by stars.](image-url)
desaturases and related enzymes. Because of the lack of sequence similarity between these families of enzymes, it is quite possible that manual identification of corresponding residues could be fortuitous and have no biological basis. We therefore tested the functional implications of converting these residues individually to alanine residues to assess their biological significance.

Previous studies on integral membrane desaturase enzymes have implicated a role for the eight-histidine residues of the conserved motif in catalysis [22,27,30]. See Fig. 1 for a comparison of the eight-histidine motif-containing regions of desaturases, hydroxylases and related enzymes. Here we test the hypothesis that the histidine motif of AlkB is related to that of the desaturases by investigating the effect of converting individual histidine residues to alanines. If the motif is performing the same function, then conversion of any of the eight alanines would be predicted to result in loss of function as it did for the desaturases. To facilitate these experiments we first inserted a number of convenient restriction enzyme sites into AlkB without altering the translated product to simplify the exchange of recombinant DNA fragments containing desired mutations. Histidine residues were individually converted to alanine residues to minimize destabilization resulting from steric effects and changes in secondary structure resulting from amino acid substitutions. All eight conserved histidines proposed comprising the conserved motif were chosen for conversion to alanine residues. Along with the eight conserved residues, three non-conserved histidine residues, each selected because they are positioned within five amino acids of a conserved histidine residue, were also chosen for comparison. Because all non-heme diiron centers characterized to date [11,20,21] contain one or more carboxylate ligand(s), we searched for candidate aspartic or glutamic acid residues that could serve this purpose. Low sequence similarity among the divergent members of the type III diiron enzymes makes sequence alignment beyond the conserved histidine regions unreliable, rendering the identification of putative carboxylate ligands speculative at best. However, we did observe an aspartic acid residue at position 181 that is partly conserved across the eukaryotic desaturase, hydroxylase, epoxygenase, acetylase, conjugase and ketolase (± one residue, see Fig. 1).

We therefore chose to investigate the effect of converting this residue to an alanine in addition to the 11 histidine residues described above.

3.1. AlkB expression, membrane isolation and enzyme quantification
To determine the effects of site-specific mutations within the AlkB enzyme, mutants were individually constructed and expressed in E. coli. Membrane fractions greatly enriched in AlkB protein were isolated. The accumulation of AlkB within the isolated membrane fractions was somewhat variable (over approximately a 5-fold range). Though there was some batch-to-batch variability for AlkB accumulation in membranes of cells expressing particular mutants, some mutants, e.g. D181A and H315A (Fig. 2A, lanes 7 and 9 respectively), showed consistently decreased accumulation. For the purposes of AlkB assay therefore, we scanned the AlkB bands within gels loaded for equal total protein and determined their AlkB content. Fig. 2B shows the membrane preparations shown in A, loaded on an equal AlkB content basis. This quantification was used as the basis for AlkB activity determinations described below.

3.2. Activity determination for AlkB and mutants thereof
Consistent with earlier results for the rat Δ'-desaturase [22] and Δ'-sterol-desaturase [27], conversion of histidine residues to alanines at AlkB positions 138, 142, 168, 172, 312, 315, and 316, which comprise the conserved eight-histidine motif, rendered the enzyme inactive (within the detection limits of the assay of <1% of wild-type activity; see Table 1 for a summary of the specific activity of mutant AlkB enzymes). In contrast, conversion of three non-conserved histidine residues at positions 163, 308 and 318, that are each located within five residues of a conserved histidine, resulted in partial loss of activity to 54, 33, and 28% of that for wild type, respectively. Conversion of the aspartate residue at position 181 to an alanine residue resulted in the loss of any detectable activity.

4. Discussion
Previous Mössbauer experiments have suggested the presence of an antiferromagnetically coupled diiron cluster at the active site of AlkB that participates in catalysis [16]. However,
none of the integral membrane diiron proteins that perform oxygen-dependent reactions on inactivated substrates shown in Fig. 1 contains the conserved amino acid motif ([D/E X₂ H₃]) characteristic of the soluble diiron proteins, suggesting the membrane enzymes possess a different coordination environment [22,25]. The identification of a residue that satisfies the requirements for the conserved eight-histidine motif common desaturases, epoxidases, acetylenases, conjugases, ketolases, decarboxylases and methyl oxidases raised the possibility that these residues could perhaps include the iron-binding ligands. However, the lack of detectable sequence similarity between AlkB and the desaturase family of enzymes prevents the direct interpretation of these residues within AlkB without experimental evidence for their requirement for catalysis. This study clearly shows that these eight residues are required for catalysis. Further, it shows that the effect is specific for the histidines that make up the motif because three histidines located within five amino acids of the conserved histidines were all functional. These data are consistent with the hypothesis that the histidines include ligands to the diiron center. Additional support for this hypothesis comes from (i) the fact that the histidine motif represents the only common sequence signature detected amongst these widely divergent proteins; (ii) that the tripartite sequence is found in the same order and with similar spacing amongst all members of the group; (iii) that the three histidine-containing regions are present in equivalent hydrophilic regions adjacent to equivalent hydrophobic regions that were experimentally defined as transmembrane domains in AlkB [29]; (iv) that the three regions of the histidine motif are present on the cytoplasmic face of the membrane for AlkB [29]; (v) that all the proteins comprising this group perform high energy oxidation chemistry on unactivated substrates (or closely related compounds) [25]; and (vi) that there is precedent for diiron clusters coordinated primarily by histidine residues, i.e. hemerythrin and myohemerythrin [18]. The mutational evidence on AlkB presented in this study is consistent with the hypothesis that these residues are involved in coordination of the diiron center, because substitution of any of the eight conserved histidines resulted in complete loss of measurable activity, while substitution of three adjacent non-conserved histidine residues resulted in modest reductions of activity compared to those of wild type.

The importance of the histidine motif was previously established for three divergent integral membrane desaturases that share no overall sequence similarity to AlkB; from rat, Synechocystis, and Arabidopsis [22,27,30]. While the rat and Synechocystis studies on acyl lipid desaturases contributed to establishing the importance of the conserved histidine motif, neither of them could be considered definitive. For the rat–yeast complementation study, the assay sensitivity was poorly defined because the threshold-specific activity of a mutant enzyme required for complementation is unknown. Thus, for enzymes that complement, an indication of activity could be inferred from the level of accumulation of unsaturated fatty acids, but for enzymes that fail to complement no activity information could be gained [22]. The Synechocystis analysis was incomplete in that only four of the eight conserved residues were investigated [30]. In contrast, the Arabidopsis study on a distantly related sterol-C5(6)-desaturase was more definitive, with all eight histidine residues individually shown to be essential for function by both in vivo and in vitro assays [27].

Additional evidence for the importance of the eight-histidine motif comes from studies on the plant Fad2-like enzymes, specifically one desaturase and another closely related hydroxylase enzyme [34]. The experiments were designed to identify factors that lead to the partitioning of functional outcome between desaturation and hydroxylation. Based on a comparison of seven desaturase sequences with two independently evolved hydroxylase sequences, seven amino acids were initially identified as candidates for outcome determination. When the equivalent residues from the oleate 12-hydroxylase were substituted for the residues at equivalent positions in an oleate 12-desaturase, the desaturase took on hydroxylase character [34]. Further investigation revealed that residues 148 and 324 of Fad2, residues positioned three and five residues from histidines within regions Ib and II respectively, accounted for almost all of the observed change in activity, suggesting that they abut the active site [35]. Consistent with this interpretation, changes were effective when reciprocal substitutions were made (i.e. when residues from the desaturase were introduced into equivalent positions in the hydroxylase) implying that substitution of larger for smaller residues and smaller for larger residues occurred without mutually exclusive steric clashes. This could most easily be explained if the residues abut the active site substrate-binding pocket [34]. Taken together these data overwhelmingly support the hypothesis that this diverse class of membrane enzymes has a diiron center with histidine ligands at its active site.

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