Spectroscopic and magnetic studies of wild-type and mutant forms of the Fe(II)- and 2-oxoglutarate-dependent decarboxylase ALKBH4

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The Fe(II)/2OG (2-oxoglutarate)-dependent dioxygenase super-family comprises proteins that couple substrate oxidation to decarboxylation of 2OG to succinate. A member of this class of mononuclear non-haem Fe proteins is the Escherichia coli DNA/RNA repair enzyme AlkB. In the present work, we describe the magnetic and optical properties of the yet uncharacterized human ALKBH4 (AlkB homologue). Through EPR and UV–visible spectroscopy studies, we address the Fe-binding environment of the proposed catalytic centre of wild-type ALKBH4 and an Fe(II)-binding mutant. We could observe a novel unusual Fe(III) high-spin EPR-active species in the presence of sulfide with a g\text{max} of 8.2. The Fe(II) site was probed with NO. An intact histidine-carboxylate site is necessary for productive Fe binding. We also report the presence of a unique cysteine-rich motif conserved in the N-terminus of ALKBH4 orthologues, and investigate its possible Fe-binding ability. Furthermore, we show that recombinant ALKBH4 mediates decarboxylation of 2OG in absence of primary substrate. This activity is dependent on Fe as well as on residues predicted to be involved in Fe(II) co-ordination. The present results demonstrate that ALKBH4 represents an active Fe(II)/2OG-dependent decarboxylase and suggest that the cysteine cluster is involved in processes other than Fe co-ordination.

Key words: AlkB, AlkB homologue (ALKBH4), EPR, non-haem Fe, UV–visible spectroscopy.

INTRODUCTION

The superfamily of Fe(II) and 2OG (2-oxoglutarate, also known as \(\alpha\)-ketoglutarate)-dependent dioxygenases (Pfam accession number PF03171) is the largest known non-haem Fe protein family able to carry out hydroxylation reactions of unactivated C–H groups. These enzymes act on a variety of substrates [1,2], and the reaction occurs by reductive activation of molecular oxygen coupled with decarboxylation of the co-substrate 2OG to succinate [3–5]. Within the oxidation process through an Fe(IV)O intermediate, one of the oxygen atoms from O2 is incorporated into the succinate moiety and the other becomes a hydroxy group in the product [3–5]. The catalytically active site is formed by a mononuclear non-haem Fe centre co-ordinated, in general, by two histidine residues and one carboxylate moiety [6–9]. This site is responsible for the binding of 2OG as well as dioxygen. During turnover, the Fe(II) hexa-co-ordinated states change to a final penta-co-ordinated state with an open oxygen co-ordination site [6–9], as observed in the three-dimensional structure of the deacetoxycephalosporin C synthase with Fe(II) and 2OG bound (PDB code 1RXG), the first structurally characterized member of this family [10]. However, when uncoupled turnover of 2OG takes place, either in the absence of natural substrates or due to incorrect orientation of substrates in the active site, decomposition of 2OG into succinate and CO2 may lead to enzyme deactivation.

The Fe(II)/2OG dioxygenase AlkB from Escherichia coli is a repair enzyme, which is induced as part of the adaptive response to alkylation damage. AlkB catalyses demethylation of 1meA (1-methyladenine) and 3meC (3-methylcytosine) in DNA in a reaction where the methyl group is hydroxylated and then released as formaldehyde, thereby regenerating the normal base [11,12]. Additionally, AlkB repairs the structurally analogous lesions 1meG (1-methylguanine) and 3meT (3-methylthymidine) and the bulkier exocyclic etheno and etheno adducts [13–17]. Moreover, AlkB also displays activity on methylated RNA [18].

A bioinformatics analysis revealed eight different mammalian ALKBHs (AlkB homologues), denoted ALKBH1–8 [19], and a more recent study has demonstrated FTO (fat mass and obesity-associated protein) [20] to be a functional ALKBH and thus the ninth member of this family [21]. AlkB-like in vitro repair activities have been reported for ALKBH1 [22], ALKBH2, ALKBH3 [23] and FTO [21], but only ALKBH2 has been convincingly demonstrated to function as a repair enzyme in vivo [24,25].

Recently, it has become clear that the ALKBH proteins are involved in processes other than DNA/RNA repair, as ALKBH8 has been demonstrated to be a tRNA modification enzyme [26–28] and ALKBH1 has been implicated in gene regulation [29]. ALKBH4/ALKBH7 remain completely uncharacterized; in fact, nothing is known about their ability to bind Fe-metal ion, the nature of the so-formed active site(s) and their optical/magnetic fingerprints. From the sequence alignment of putative ALKBH4 orthologues from various organisms, we noted the presence of a cluster of four highly conserved cysteine residues very close to the N-terminus (Figure 1). This cluster is not present in AlkB, and we reasoned that it potentially constitutes a complementary Fe–S binding site. In order to clearly elucidate the nature of the Fe-metal binding by human ALKBH4 in its anticipated binding triad of two histidine residues and one carboxylate motif (His\textsuperscript{169}–Asp\textsuperscript{171}, His\textsuperscript{254}) (Figure 2) and to address the possible role of the cysteine cluster as an additional Fe–S centre, in the present work we studied the enzymatic activities and the optical and magnetic fingerprints.
Figure 1  Sequence alignment of the N-terminal part of putative ALKBH4 orthologues from different organisms

Arrows indicate the cysteine residues subjected to mutagenesis (C15A/C17A). GenBank® Identifier (gi) numbers of the displayed sequences are as follows: 8923019 (Homo sapiens), 81905645 (Mus musculus), 68372246 (Danio rerio), 24583140 (Drosophila melanogaster) and 25148697 (Caenorhabditis elegans). The alignment was generated using the MAFFT algorithm [48] and displayed in Jalview [49].

Figure 2  Close-up view of the catalytic centre of AlkB in the presence of bound 2OG (PDB code 2FD8) [50]

The amino acid residues that complex the Fe metal are indicated in black (H131, D133, H187). A water molecule (W) weakly interacting with the Fe ion (Fe–H2O = 2.9 Å) is also shown. The amino acid residues of ALKBH4 thought to form a similar Fe metal-binding triad (H169, D171, H254) [19] are indicated by boxed magenta labels.

[MATERIALS AND METHODS]

Plasmid construction and site-specific mutagenesis of ALKBH4

The cDNA encoding human ALKBH4 was amplified by PCR and inserted between the NdeI and BamHI sites of the expression vector pET-28a (Novagen). The QuikChange™ Site-Directed Mutagenesis Kit (Stratagene) was used for generation of ALKBH4 mutants. To generate the pET-H169A/D171A plasmid, the primers 5′-CTGCCATAGCACCCTGGC-GCCAGGCGCGG-3′ and 5′-GGTCGGATGCCTTGGC-ACCAGCTTCGAGGACTTCG-3′ were used for generation of the plasmid pET-C15A/C17A (underlining indicates mutation sites). Plasmids encoding GST (glutathione transferase)-tagged mutants were obtained by subcloning the mutated cDNA fragments into the vector pGEX-6P-2 (GE Healthcare), resulting in the plasmids pGEX-H169A/D171A and pGEX-C15A/C17A respectively (BamHI and EcoRI sites were used for H169A/D171A, and BamHI and SalI sites were used for C15A/C17A). All PCR-generated fragments were verified by DNA sequencing.

Purification of ALKBH4 and mutant proteins

Plasmids were transformed into the E. coli strain BL21-CodonPlus(DE3)-RIPL (Stratagene), and protein expression was induced by the addition of 0.075–0.1 mM IPTG (isopropyl β-D-thiogalactopyranoside). Cells were disrupted by French press treatment [two passages through a standard FRENCH® pressure cell (Thermo Electron) at a working pressure of 1000 psi (1 psi = 6.9 kPa)], and proteins were purified from clarified lysates using affinity purification. For spectroscopic analysis, ALKBH4 and mutants were expressed as GST-fusion proteins and purified using glutathione-Sepharose 4B medium (GE Healthcare). The GST tag was removed by on-column enzymatic cleavage using PreScission protease (GE Healthcare), and HiTrap Desalting columns (GE Healthcare) were used for buffer exchange (50 mM Hepes, pH 7.0, and 100 mM KCl). His6-tagged AlkB proteins for decarboxylation assays were purified using TALON® Metal Affinity Resin (Clontech). Proteins were analysed by SDS–4–12% PAGE and subsequent Coomassie Brilliant Blue staining. The metal content (Fe) in the protein preparations was probed by ICP-AEP (inductively coupled plasma atomic emission spectroscopy), with detection at 238.204 nm for the Fe ion emission line.

Assay for decarboxylation of 2OG to succinate

The reactions were performed as described previously [14]. Briefly, reaction mixtures of 50 μl contained 50 mM Hepes, pH 7.5, 4 mM ascorbic acid, 160 μM 2OG (10% [5–14C]2OG), 80 μM FeSO4 (when indicated) and His6-tagged ALKBH4 (500 pmol of wild-type or mutants) or E. coli His–AlkB (100 pmol). EDTA (1 mM) was added to samples without FeSO4. Reactions were incubated at 37°C for 30 min. The amount of [1–14C]succinate formed was measured by scintillation counting after precipitation of the remaining 2OG with 2,4-DNPH (2,4-dinitrophenylhydrazine).
Metal-reconstitution procedures and spectroscopic measurements

Concentrated (<300 μM) protein solutions were obtained by centrifugal filter devices (Microcon YM-10) in 50 mM Hepes, pH 7.0, and were then made anaerobic in airtight vessels by several rounds of vacuum treatment and argon exchange using the Schlenk technique [29a]. The minimum amount (<2 mol per mol of protein) of a freshly prepared anaerobic solution of Na₂S₂O₄ (5.7 mM in 50 mM Hepes, pH 7.0) was then added to the protein solutions using airtight syringes. Stoichiometric amounts of Fe(II) ammonium sulfate were added to the protein samples in a similar way, using anaerobic stocks. In some cases, small amounts (~20 μM) of Methyl Viologen were added in order to monitor that strict anaerobic conditions were maintained within reconstitution procedures. Anaerobic solutions of 2OG and Na₂S in 50 mM Hepes, pH 7.0, were freshly prepared and added to the protein samples at a 10-fold excess. These solutions were transferred to quartz EPR tubes through airtight syringes that were maintained under argon, and then the tubes were sealed with Precision Seal® rubber septa (Sigma–Aldrich). Nitric oxide (NO) gas was added through airtight syringes directly into the quartz EPR tubes containing the sample protein, or to airtight 1 cm optical path quartz cuvettes. Samples were maintained at cold temperatures (≤10°C). Sample oxidation was obtained by air exposure followed by slow injection of O₂ (~90% purity, ~5 min). In the experiments employing reconstitution in the presence of 2OG/Na₂S/Na₂S₂O₄, sample oxidation was obtained by injection of 50 μl of O₂-saturated 50 mM Hepes, pH 7.0, into EPR tubes containing 100 μl of protein sample, followed by fast quenching in liquid nitrogen. The low-temperature EPR spectra were acquired on a Bruker Elexsys 560 instrument equipped with a dual-band X-resonator (Bruker) and by using a ESR900 He-flow cryostat (Oxford Instruments). UV–Vis spectra were recorded on a HP 8452A diode-array spectrophotometer (Hewlett Packard).

RESULTS AND DISCUSSION

Decarboxylation activity of ALKBH4 and mutant proteins towards 2OG

Like several of the Fe(II)/2OG dioxygenases, E. coli AlkB possesses the ability to perform decarboxylation of 2OG to succinate even in the absence of primary substrate [11,12]. Since the primary substrate of ALKBH4 is still unknown, we took advantage of such uncoupled co-substrate conversion to test the in vitro enzymatic activity of ALKBH4. For this purpose, recombinant His₆-tagged ALKBH4 and two mutants were purified from E. coli (Figure 3A) and subsequently assayed for 2OG decarboxylation activity. Indeed, ALKBH4 was able to catalyse succinate formation in the presence of the co-factor Fe(II), although not to the same extent as AlkB (Figure 3B). The decarboxylation activity was reduced to background levels when Fe(II) was not included, demonstrating the requirement for Fe, which is indicative of AlkB proteins. Likewise, the H169A/D171A mutant was devoid of the ability to perform 2OG turnover. The C15A/C17A mutant displayed a decarboxylation activity similar to that of the wild-type ALKBH4 protein, suggesting that the cysteine motif is not involved in Fe(II) coordination. Taken together, these results experimentally confirmed that ALKBH4 is a Fe(II)/2OG-dependent decarboxylase.

Spectroscopic fingerprints of the Fe catalytic centre of ALKBH4 and mutant proteins probed by EPR

The electronic/magnetic fingerprints of the native and mutant proteins were then addressed by low-temperature EPR measurements down to cryogenic temperatures. We probed different conditions where the metal co-ordination environment of Fe reconstituted ALKBH4, and mutant proteins were analysed both in the reduced [Fe(II)] and oxidized [Fe(III)] states, as well as in the presence or absence of 2OG/succinate. The results are summarized in Table 1. It should be noted that the isolated proteins (ALKBH4 and mutants), even when kept in iced-cooled solutions (4°C), aggregated and then precipitated at concentrations higher than 0.5 mM. The process was accelerated greatly when an excess (e.g. 5-fold) of dithionite was added to the solution when anaerobic Fe(II)-reconstitution procedures were carried out. Nevertheless, low protein concentrations (<0.3 mM) and a slight excess of Na₂S₂O₄ (approx. twice greater) allowed efficient reconstitution of the apo-proteins with the Fe(II) metal ion, hence avoiding precipitation. The purified ALKBH4 protein, in the absence of added cofactors, gave rise to an extremely weak high-spin Fe(III) signal at g ≈ 4.3 (Figure 4A). This resonance did not increase upon addition of oxidants (e.g. Na₂IrCl₄). This agrees with the ICP-AES findings that indicated the presence of Fe traces only in the purified protein. Traces of Cu(II) ion as an impurity (around g ≈ 2.1) were also detected, with relative amounts that changed from sample to sample. When ALKBH4 and the C15A/C17A and H169A/D171A mutants were incubated with stoichiometric amounts of Fe(II) under reducing conditions, either in the presence or absence of 2OG/succinate, the recorded
low-temperature EPR spectra did not show any Fe-related signal (Figure 4B). It is important to note that the only resonance signal emerging in the spectra was always associated with the presence of MV•+ (Methyl Viologen radical cation) ($g = 2.00$ in Figure 4B), which was used as an internal indicator to probe the attainment of rigorous anaerobic conditions. These results can be interpreted at X-band EPR with either (i) an integer-spin system having a high-spin Fe(II) state ($S = 2$), where $S$ is the ground-state spin characterized by a large axial ZFS (zero-field splitting) term called $\delta$ in EPR or $\Delta$ in MCD (magnetic CD), as it occurs in many mononuclear non-haem Fe proteins where such configuration is usually not spectroscopically accessible, or alternatively (ii) by a low-spin Fe(II) state ($S = 0$) [1,2,30]. NO has been successfully used as a probe for such centres [31], since it can convert the $S = 2$ species into an EPR-active $S = 3/2$ species, as observed, for example, in IPNS (isopenicillin N synthase) [32], in extradiol-cleaving catechol dioxygenases such as 2,3-CTD (catechol 2,3-dioxygenase) and 4,5-PCD (protocatechuate 4,5-dioxygenase) [33,34], as well as in non-haem Fe model complexes [35]. Upon anaerobic addition of NO to Fe(II)-reconstituted ALKBH4 and C15A/C17A protein in the absence of 2OG, a strong axial EPR signal centred at a $g_{xx}$ of 3.97 developed ($g$-tensor components at $g_1 = 4.08$, $g_2 = 3.97$ and $g_3 = 2.00$), together with a second asymmetric resonance at $g \approx 2.00$ (Figure 4C). The latter signal contained features characteristic of a small amount of NO radical being trapped in the frozen matrix solution. Similarly, when anaerobic reconstitution of the ALKBH4 and C15A/C17A proteins was carried out with both Fe(II) and a 10-fold excess of 2OG, a strong axial resonance signal centred at $g_{xx} = 3.97$ developed after NO addition, being accompanied by a small highly asymmetric signal around $g \approx 2.00$ (Figure 4E). Analogous spurious signals at $g \approx 2.00$ are found in other non-haem Fe proteins, such as 4,5-PCD [36] and IPNS [32]. Therefore in both cases a nearly axial $S = 3/2$ Fe(II)–NO adduct formed with the $E$ (rhombic) versus $D$ (axial) term [$|E/D|/|D| \approx 0.010$].

Similarly, anaerobic reconstitution of the proteins with both Fe(II) and a 10-fold excess of the product succinate, followed by addition of NO, led to a single strong resonance signal around $g_{xx} = 3.93$ that contained a slightly higher degree of rhombicity ($|E/D|/|D| \approx 0.015$) (Figure 4F). The H169A/D171A protein behaved very differently. After being incubated with Fe(II) under anaerobic conditions, a very strong signal centred at $g \approx 2$ appeared immediately after addition of NO. Only a very weak resonance around $g \approx 4$ could be detected, with small variations in relative intensities from preparation to preparation. These findings confirm that when the histidine-carboxylate Fe-binding region is abrogated, only a much smaller fraction of Fe productively forms stable $S = 3/2$ Fe–NO complexes (Figure 4D).

Upon addition of oxygen, the ALKBH4 protein sample that contained 2OG (Figure 4H), and also the sample that was deficient in co-substrate (Figure 4I), displayed formation of strong similar signals with broad shoulders at $g \approx 4.26$, being accompanied by the appearance of weak resonances at $g_{xx} = 9.40$. Those signals originate from transition involving the middle and ground Kramer’s doublets respectively and are typically found in high-spin rhombic Fe(III) centres ($|E/D|/|D| \approx 0.25$). The same resonances developed in the C15A/C17A mutant upon Fe(II) oxidation. Similarly, a strong signal formed at $g_{xx} = 4.28$ upon oxidation of the Fe(II) H169A/D171A mutant (Figure 4G). However, the presence of broad shoulders like those of the Fe(III) forms of both ALKBH4 and the C15A/C17A mutant were not detected. Such EPR resonance can therefore be interpreted as arising from spurious or non-specifically bound Fe(III) metal ion. Surprisingly, addition of oxygen to the metal-reconstituted ALKBH4 and C15A/C17A proteins in the presence of succinate resulted in protein precipitation. The reason for this is presently unknown.

Motivated by the possibility that the N-terminal cysteine-rich motif might provide an Fe–S cluster, we measured the EPR spectrum of ALKBH4 in the presence of inorganic $S$ and Fe(II).
Anaerobic incubation of ALKBH4 with 2OG and Fe(II) in the presence of Na2S (10-fold excess), followed by addition of O2 and fast quenching at liquid-N2 temperature (77 K, −196°C), resulted in a transient EPR-active species that exhibited resonances at \( g_{\text{eff}} = 8.24, 5.56 \) and 4.26 (Figure 5). Upon thawing, this new species relaxed fast (≈20 s) to the high-spin rhombic Fe(III) ion with |\( E / D \)\| ≈ 0.25, showing resonances similar to those described in Figure 4(H). Identical behaviour was observed for the C15A/C17A mutant, but not for the mutant H169A/D171A. These effects suggest that the structural changes occurring in the active site within the Fe(II)→Fe(III) oxidation process are modulated further by the presence of Na2S. It is important to note that similar signals and a similar decay process have been reported in the non-haem Fe(III) centre present in photosystem II, for example, decrease of the \( g_{\text{eff}} = 8.07 \) and \( g_{\text{eff}} = 5.58 \) signals being accompanied by induction (increase) of the signal at the \( g_{\text{eff}} = 4.26 \) signal, as a result of UV-B irradiation (280–320 nm) \([37,38]\). These types of resonances have previously been analysed by Weisser et al. \[39\] in terms of ZFS-distributed S = 5/2 systems in Fe–catecholato complexes containing moderate rhombic distortion (|\( E / D \)\| ≈ 0.12). Furthermore, EPR spectra similar to those reported in Figure 5, although not transient, are observed in Fe(III)–catecholato complexes as model systems of intradiol-cleaving catechol dioxygenases \[40\], in PAH (phenylalanine hydroxylase) in the presence of catecholamine feedback inhibitors or even when recording PAH in Tris buffer \[41,42\].

The optical signatures of ALKBH4 and mutant proteins probed by UV–Vis absorption spectroscopy

In parallel with the low-temperature EPR experiments, the optical fingerprints of the various forms of ALKBH4 and mutants [Fe(II)/Fe(III)] in the presence of, and without, 2OG were probed by UV–Vis absorption spectroscopy. The electronic spectrum of the purified ALKBH4 protein did not show absorption features in the visible region (Figure 6A, dashed line). Similarly, the C15A/C17A and H169A/D171A proteins gave featureless spectra. Upon reconstitution with Fe(II), but in the absence of 2OG, both ALKBH4 and the C15A/C17A mutant developed a broad and composite absorption band, extending from 400 nm (\( \lambda = 404 \text{ nm, } \varepsilon \approx 570 \text{ M}^{-1} \cdot \text{cm}^{-1} \)) to 600 nm (\( \lambda = 490 \text{ nm, } \varepsilon \approx 260 \text{ M}^{-1} \cdot \text{cm}^{-1} \)) (Figure 6A, dashed and dotted line). However, the strong absorption band around 320 nm, due to the presence of Na2S2O4 (reducing equivalents) in the medium, masked an additional high-energy absorption signature, around 350 nm (\( \varepsilon \approx 920 \text{ M}^{-1} \cdot \text{cm}^{-1} \)), as substantiated in those samples reconstituted with Fe(II) without the supply of reducing agents (Figure 6A, continuous line). When a 10-fold excess of 2OG was added under reducing conditions, a slightly different chromophore (\( \lambda = 430 \text{ nm, } \varepsilon \approx 440 \text{ M}^{-1} \cdot \text{cm}^{-1} \)) developed quickly in both ALKBH4/Fe(II) and the C15A/C17A/Fe(II) protein (Figure 6B, dotted line), with an absorption envelope similar to that observed by Henshaw et al. \[43\] in E. coli AlkB in the presence of Fe(II)/2OG. Upon oxidation, both ALKBH4/Fe(II) and the C15A/C17A/Fe(II) mutant showed the formation of an

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Table 1 X-band EPR data obtained at \( T = 8.0 \pm 0.5 \text{ K} \) for ALKBH4 and mutant proteins (H169A/D171A and C15A/C17A), in their purified forms, after Fe-metal reconstitution in the presence or absence of 2OG/succinate and upon interaction with NO

| Protein | Observed EPR g-values | \(|E / D\)| | S | Notes |
|---------|-----------------------|------|----|------|
| ALKBH4 purifieda, b | 4.30 [Fe(III)], 2.10 [Cu(II)] | 1/3 | 5/2 [Fe(III)], 1/2 [Cu(II)] | Fe(III) and Cu(II) traces |
| ALKBH4/Fe(II)a, b | 2.00 | n.d. | 2 [Fe(II)], 1/2 (MV**- ) | Recorded under reducing conditions (Na2S2O4) in the presence of MV**- |
| ALKBH4/Fe(II)/NOa | 4.08, 3.97, 2.00 | 0.010 | 3/2 [Fe(II)–NO complex] | Recorded under reducing conditions (Na2S2O4) | NO radical |
| ALKBH4/Fe(II)/caffeic acid | 4.08, 3.97, 2.00 | 0.015 | 3/2 [Fe(II)–NO complex] | Recorded under reducing conditions (Na2S2O4) | NO radical |
| H169A/D171A/Fe(II)/NO | ~4.00, 2.02 | n.d. | 3/2 [Fe(II)–NO complex], 1/2 (NO radical) |
| ALKBH4/Fe(III)/2OG | 8.24, 5.56, 4.26, 2.10 | 0.120 | 5/2 [Fe(III)], 1/2 (Cu(II)) | Protein precipitation |
| ALKBH4/Fe(III)a | 9.40, 4.26, 2.10 | 0.250 | 5/2 [Fe(III)], 1/2 (Cu(II)) | Cu(II) was a minor species |
| ALKBH4/Fe(III)a | 4.28, 2.10 | 1/3 | 5/2 [Fe(III)], 1/2 (Cu(II)) |
| ALKBH4/Fe(III)/2OGa | 4.28, 4.26, 2.10 | 0.250 | 5/2 [Fe(III)], 1/2 (Cu(II)) |
| ALKBH4/Fe(III)/2OGa | n.d. | n.d. | n.d. | Non-specifically bound Fe(III) |

aThe same features were observed for the C15A/C17A mutant protein.
bThe same features were observed for the H169A/D171A mutant protein.

Figure 5 Transient EPR spectra of ALKBH4 recorded within the Fe(II)→Fe(III) oxidation process

X-band EPR spectra of ALKBH4 (120 μM) in 50 mM Hepes, pH 7.0) reconstituted with stoichiometric amounts of Fe(II), 2OG (10-fold excess) and Na2S (10-fold excess), recorded after addition of O2 followed by quenching after \( t = 5 \text{ s} \) (upper trace) or \( t = 10 \text{ s} \) (lower trace) of the ice-cooled solutions down to liquid-N2 temperature \( (T = 77 \text{ K}) \). The measurements were performed at \( T = 8.0 \pm 0.5 \text{ K} \); frequency, 9.67 GHz; modulation frequency, 100 KHz; modulation amplitude, 0.7 mT; gain, 55 dB; time constant, 81.92 ms; sweep time, 323 s; microwave power, 0.8 mW; cavity quality factor \( Q = 4200–4400 \); two scans were accumulated and averaged.

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Figure 6 UV–Vis absorption spectroscopy of Fe-reconstituted ALKBH4

(A) Purified ALKBH4 (dashed line), ALKBH4/Fe(II) recorded under anaerobic conditions in the absence of Na2S2O4 (dotted line), and ALKBH4/Fe(II) recorded under anaerobic conditions in the presence of Na2S2O4 (dotted line) and after oxidation (continuous line). Note that the spectrum showing the optical feature of purified ALKBH4 (dashed line) from (A) is also included here for easier comparison.

(B) ALKBH4/Fe(II)/2OG recorded under anaerobic conditions in the presence of Na2S2O4 (dotted line) and after oxidation (continuous line). The corresponding spectrum of the H169A/D171A mutant (incubated with Fe(II) and 2OG) after NO exposure under anaerobic conditions is shown as a dashed line. Protein concentrations were 0.22 mM in 50 mM Hepes, pH 7.0, cooled solutions (T ≤ 10°C). Abs, absorbance.

absorption band centred at 530 nm (ε ≈ 920 M⁻¹·cm⁻¹), being accompanied by the appearance of a shoulder at higher energy (λmax = 370 nm, ε ≈ 2000 M⁻¹·cm⁻¹) (Figure 6A, continuous line). Similarly, upon oxidation of Fe(II)- and 2OG-reconstituted ALKBH4 and C15A/C17A protein, an absorption band that was slightly blue-shifted (λmax = 520 nm, ε ≈ 860 M⁻¹·cm⁻¹) was detected together with a shoulder at λmax = 404 nm (ε ≈ 1580 M⁻¹·cm⁻¹) (Figure 6B, continuous line). None of the above-reported absorptions were observed in the H169A/D171A mutant, neither in the Fe-reduced nor -oxidized state. Reconstitution of ALKBH4 and the C15A/C17A protein with a 5-fold excess of Fe(II) metal ions prior to oxidation did not result in any further increase of the absorption features in the visible regions. These findings reinforce the hypothesis that (i) ALKBH4 is mononuclear in Fe content, (ii) the cysteine-rich N-terminal site does not provide an additional binding centre for the Fe(II)/Fe(III) metal ion, and furthermore (iii) an intact histidine-carboxylate site is necessary for productive Fe binding, in agreement with the results obtained in the 2OG-decarboxylation-activity assay. Since the optical features of both ALKBH4 and the C15A/C17A protein are clearly modulated by the presence of 2OG, these differences may indeed mirror modifications in the Fe co-ordination environment upon interaction with the substrate, such as the displacement of previously co-ordinated water molecules to the metal Fe. In the present study, the development of rather strong chromophores around 520–530 nm in the Fe(III) ALKBH4 and C15A/C17A protein forms closely resemble the optical spectra observed in synthetic models of non-Fe(III)–catecholato/phenolato complexes [40]. Therefore the spectroscopic behaviour displayed especially for the Fe(III) forms of ALKBH4 and the C15A/C17A mutant is different from that previously observed for analogous non-Fe proteins, such as AlkB and the Fe(II)/2OG-dependent TauD (taurine dioxygenase). AlkB exhibits an absorption band at λmax = 560 nm (ε = 1258 M⁻¹·cm⁻¹) [44] or, as found in other reports [12,43], a much weaker broad band at 450–500 nm, whereas TauD shows an absorption band at λmax = 530 nm (ε = 140 M⁻¹·cm⁻¹) [45], when both 2OG and Fe(II) are present under strict anaerobic conditions. In these proteins, exposure to oxygen in the absence of primary substrate causes the development of greenish-brown chromophores. In TauD (λmax = 550 nm, ε = 700 M⁻¹·cm⁻¹), the absorption envelope in the oxidized protein arises from a self-hydroxylation reaction of a tyrosine residue (Tyr²⁸) near the non-Fe metal site [45]. In AlkB, the formation of a greenish-brown chromophore upon oxidation (λmax = 595 nm, ε = 960 M⁻¹·cm⁻¹) indicates a similar self-hydroxylation process that involves Trp¹⁷⁸ [43]. In order to further study the O₂-binding properties of ALKBH4, we followed the optical changes of the Fe(II) metal core upon interaction with the dioxygen analogue NO, as performed in the EPR experiments reported earlier. Addition of NO to an anaerobic solution of ALKBH4 containing Fe(II), 2OG and Na2S2O4 produced a strong yellowish-green chromophore, with a broad absorption band around 580 nm (ε ≈ 620 M⁻¹·cm⁻¹) being accompanied by a more intense peak at 436 nm (ε ≈ 2430 M⁻¹·cm⁻¹) (Figure 6C, continuous line). A similar spectrum was obtained for the C15A/C17A mutant (results not shown). These optical features closely resemble those reported for other non-Fe proteins [46] anaerobically treated with NO, such as PAH (λmax = 440 nm, ε ≈ 2300 M⁻¹·cm⁻¹) in the presence of its co-substrate tetrahydropterin [47], which exhibits, in addition, similar EPR resonance to our NO complex. However, after injection of oxygen, the Fe(II)–NO adduct decayed into a greenish-brown chromophore (λ = 580 nm, ε ≈ 620 M⁻¹·cm⁻¹, and λ = 770 nm, ε ≈ 270 M⁻¹·cm⁻¹) (Figure 6C, dotted line), without recovery of the optical features for the Fe(III) form shown previously (Figure 6B, continuous line). This might indicate that the protein becomes more sensitive towards side...
Conclusions

In the present work, we have addressed the characteristics (electronic and magnetic) of the Fe metal core in the human AlkB homologue ALKBH4, and we have shown that an intact His<sup>169</sup>-Asp<sup>171</sup>-His<sup>254</sup> motif is necessary for productive Fe binding and for decarboxylation activity towards 2OG. Furthermore, we have shown that the N-proximal cysteine-rich motif of ALKBH4 is not involved in Fe binding. Although the biological function of ALKBH4 remains elusive, its ability to bind and decarboxylate 2OG after metal reconstitution, together with the optical and spectroscopic fingerprints of its suggested catalytic centre, confirm for the first time that ALKBH4 truly belongs to the class of 2OG-dependent mononuclear non-haem Fe proteins. Additional work is needed to determine the nature of the transient species detected within the reoxidation process in the presence of Na<sub>c</sub>S.

AUTHOR CONTRIBUTION

Linn Bjernstam planned experiments, produced wild-type and mutant proteins, undertook activity measurement, analysed all of the results and wrote the manuscript. Giorgio Zoppellaro planned experiments, performed EPR and light-absorption experiments, analysed all of the results and wrote the manuscript. Anne Tomter performed EPR experiments, analysed results and wrote the manuscript. Pål Falnes designed wild-type protein and mutants, provided experience in AlkB protein, analysed all of the results and wrote the manuscript. Kristoffer Andersson planned physical chemistry experiments, provided experience in non-haem Fe proteins, analysed all of the results and wrote the manuscript.

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REFERENCES

1. Costas, M., Mehn, M. P., Jensen, M. P. and Qin, J. L. (2004) Dioxygen activation at mononuclear nonheme iron active sites: enzymes, models, and intermediates. Chem. Rev. 104, 939–986
2. Schoffel, C. J. and Zhang, Z. (1999) Structural and mechanistic studies on 2-oxoglutarate-dependent oxygenases and related enzymes. Curr. Opin. Struct. Biol. 9, 722–731
3. Neidig, M. L., Brown, C. D., Light, K. M., Fujimori, D. G., Nolan, E. M., Price, J. C., Barr, E. W., Bollinger, Jr. J. M., Krebs, C., Walsh, C. T. and Solomon, E. I. (2007) CD and MCD of CygC3 and taurine dioxygenase: role of the facial triad in α-KG-dependent oxygenases. J. Am. Chem. Soc. 129, 14224–14231
4. Price, J. C., Barr, E. W., Tirupati, B., Bollinger, Jr. J. M. and Krebs, C. (2003) The first direct characterization of a high-valent iron intermediate in the reoxidation of an α-ketoglutarate-dependent dioxygenase: a high-spin Fe(IV) complex in taurine/α-ketoglutarate dioxygenase (TauD) from Escherichia coli. Biochemistry 42, 7497–7508
5. Que, Jr., L., and Ho, R. Y. (1996) Dioxygen activation by enzymes with mononuclear non-heme iron active sites. Chem. Rev. 96, 2607–2624
6. Hegg, E. L. and Que, Jr. L. (1997) The 2-His-1-carboxylate facial triad – an emerging structural motif in nonmononuclear non-heme iron(II) enzymes. Eur. J. Biochem. 250, 625–629
7. Neidig, M. L., Kavana, M., Moran, G. R. and Solomon, E. I. (2004) CD and MCD studies of the non-heme ferrous active site in (4-hydroxyphenyl)pyruvate dioxygenase: correlation between oxygen activation in the extradiol and α-KG-dependent oxygenases. J. Am. Chem. Soc. 126, 4486–4487
8. Purpura, V. and Moran, G. R. (2007) The diverse and pervasive chemistries of the α-keto acid dependent enzymes. J. Biol. Inorg. Chem. 12, 587–601
9. Zhou, J., Gunisior, M., Bachmann, B. O., Townsend, C. A. and Solomon, E. I. (1998) Substrate binding to the α-ketoglutarate-dependent non-heme iron enzyme clavamine synthase 2: coupling mechanism of oxidative decarboxylation and hydroxylation. J. Am. Chem. Soc. 120, 13539–13540
10. Varelaeg, K., van Scheltinga, A. C., Lloyd, M. D., Han, T., Ramaswamy, S., Perrakis, A., Thompson, A., Lee, H. J., Baldwin, J. E., Schofield, C. J. et al. (1998) Structure of a cephalosporin synthase. Nature 394, 805–809
11. Falnes, P. O., Johansen, R. F. and Seeberg, E. (2002) AlkB-mediated oxidative demethylation reverses DNA damage in Escherichia coli. Nature 418, 176–182
12. Trewick, S. C., Henshaw, T. F., Hausinger, R. P., Lindahl, T. and Sedgwick, B. (2002) Oxidative demethylation by Escherichia coli AlkB directly reverts DNA base damage. Nature 419, 174–178
13. Delaney, J. C., Smeeister, L., Wong, C., Frick, E. L., Taghiakdzad, K., Wishnik, J. S., Drennan, C. L., Samson, L. D. and Essigmann, J. M. (2005) AlkB reverses etheno DNA lesions caused by lipid oxidation in vitro and in vivo. Nat. Struct. Mol. Biol. 12, 855–860
14. Falnes, P. O. (2004) Repair of 3-methylthymine and 1-methylguanine lesions by bacterial and human AlkB proteins. Nucleic Acids Res. 32, 6250–6257
15. Frick, L. E., Delaney, J. C., Wong, C., Drennan, C. L. and Essigmann, J. M. (2007) Alloleviation of 1,N<sub>6</sub>-ethanoamide genotoxicity by the Escherichia coli adaptive response protein AlkB. Proc. Natl. Acad. Sci. U.S.A. 104, 755–760
16. Koivisto, S., Pobins, R., Lindahl, T. and Sedgwick, B. (2004) Demethylation of 3-methylthymine in DNA by bacterial and human DNA dioxygenases. J. Biol. Chem. 279, 40470–40474
17. Mishina, Y., Yang, C. G. and He, C. (2005) Direct repair of the excocyclic DNA adduct 1,N<sub>6</sub>-ethanoamine by the DNA repair AlkB proteins. J. Am. Chem. Soc. 127, 14594–14595
18. Falnes, P. O., Klungland, A. and Asoeth, J. (2007) Repair of methyl lesions in DNA and RNA by oxidative demethylation. Neuroscience 145, 1222–1232
19. Aravind, L. and Koonin, E. V. (2001) The DNA-repair protein AlkB, EGL-9, and leprecan define new families of 2-oxoglutarate- and iron-dependent dehydrogenases. Genome Biol. 2, RESEARCH0007
20. Han, Z., Niu, T., Chang, J., Lei, X., Zhao, M., Wang, Q., Cheng, W., Wang, J., Feng, Y. and Chai, J. (2010) Crystal structure of the FTO protein reveals basis for its substrate specificity. Nature 464, 1205–1209
21. Gerken, T., Girard, C. A., Tung, Y. C., Webb, C. J., Saudek, V., Hewlstone, K. S., Yeo, G. S., McDonough, M. A., Curniflfe, S., McNeil, L. A. et al. (2007) The obesity-associated FTO gene encodes a 2-oxoglutarate-dependent nuclear acid demethylase. Science 318, 1469–1472
22. Westbye, M. P., Feyzi, E., Aas, P. A., Vagbo, C. B., Talstad, V. A., Kavli, B., Hagen, L., Sundheim, O., Akbari, M., Liabakk, N. B. et al. (2008) Human AlkB homolog 1 is a mitochondrial protein that demethylates 3-methylcytosine in DNA and RNA. J. Biol. Chem. 283, 25046–25056
23. Duncan, T., Trowick, S. C., Koivisto, P., Bates, P. A., Lindahl, T. and Sedgwick, B. (2002) Reversal of DNA alkylation damage by two human dioxygenases. Proc. Natl. Acad. Sci. U.S.A. 99, 16660–16665
24. Ringovil, J., Moeen, M. N., Nordstrand, L. M., Meira, L. B., Pang, B., Bekkelund, A., Oden, P. C., Bjelland, S., Samson, L. D., Falnes, P. O. and Klungland, A. (2008) AlkB homologue 2-mediated repair of ethanoamine lesions in mammalian DNA. Cancer Res. 68, 4142–4149
25. Ringovil, J., Nordstrand, L. M., Vagbo, C. B., Talstad, V., Reile, K., Aas, P. A., Lauritzen, K. H., Liabakk, N. B., Bjork, A., Doughty, R. W. et al. (2006) Repair deficient mice reveal mAbH2 as the primary oxidative demethylase for repairing 1meA and 3meC lesions in DNA. EBMO J. 25, 2189–2198

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26 Fu, D., Brophy, J. A., Chan, C. T., Atmore, K. A., Begley, U., Paules, R. S., Dedon, P. C., Begley, T. J. and Samson, L. D. (2010) Human AlkB homolog ABHB is a tRNA methyltransferase required for wobble uridine modification and DNA damage survival. Mol. Cell. Biol. 30, 2449–2459

27 Fu, Y., Dai, Q., Zhang, W., Ren, J., Pan, T. and He, C. (2010) The AlkB domain of mammalian ABHB catalyzes hydroxylation of 5-methoxycarbonylmethyluridine at the wobble position of tRNA. Angew. Chem. Int. Ed. 49, 1–5

28 Songe-Moller, L., van den Born, E., Leithe, V., Vaqbo, C. B., Kristoffersen, T., Krekan, H. E., Kirpekar, F., Falnes, P. D. and Klungland, A. (2010) Mammalian ALKBHB possesses tRNA methyltransferase activity required for the biogenesis of multiple wobble uridine modifications implicated in translational decoding. Mol. Cell. Biol. 30, 1814–1827

29 Pan, Z., Sikandar, S., Wilterspoon, M., Dizon, D., Nguyen, T., Benirschke, K., Wiley, C., Vranas, P. and Lipkin, S. M. (2008) Impaired placental trophoblast lineage differentiation in Alkbh 1−/− mice. Dev. Dyn. 237, 316–327

29a Tidwell, T. T. (2001) William Schlenk: the man behind the flask. Angew. Chem. Int. Ed. 39, 819–822

30 Solomon, E. I. and Zhang, Y. (1992) The electronic structures of active sites in non-heme iron enzymes: nitric oxide adducts of FeII(L)(O2CCOPh)(ClO4) complexes. Inorg. Chem. 31, 903–910

31 McCleverty, J. A. (2004) Chemistry of nitric oxide relevant to biology. Chem. Rev. 104, 403–418

32 Chen, V. J., Orville, A. M., Harpel, M. R., Frolik, C. A., Suretus, K. K., Munck, E. and Lipscomb, J. D. (1989) Spectroscopic studies of isopenicillin N synthase. A mononuclear nonheme Fe2+ oxide with metal coordination sites for small molecules and substrate. J. Biol. Chem. 264, 21677–21681

33 Arciero, D. M. and Lipscomb, J. D. (1996) Binding of 18O-labeled substrate and inhibitors to protocatechuate 4,5-dioxygenase-nitrosyl complex. Evidence for direct substrate binding to the active site Fe2+ of extradiol dioxygenases. J. Biol. Chem. 261, 2170–2178

34 Arciero, D. M., Orville, A. M. and Lipscomb, J. D. (1985) [18O]Water and nitric oxide binding by protocatechuate 4,5-dioxygenase and catechol 2,3-dioxygenase. Evidence for binding of exogenous ligands to the active site Fe2+ of extradiol dioxygenases. J. Biol. Chem. 260, 14035–14044

35 Chou, Y.-M. and Qu, J. L. (1995) Model studies of α-keto acid-dependent nonheme iron enzymes: nitric oxide adducts of Fe(II)(O2CCDOPh)Cl(4) complexes. Inorg. Chem. 34, 3270–3278

36 Arciero, D. M., Lipscomb, J. D., Huynh, B. H., Kent, T. A. and Munck, E. (1983) EPR and Mossbauer studies of protocatechuate 4,5-dioxygenase. Characterization of a new Fe2+ environment. J. Biol. Chem. 258, 14981–14991

37 McEvoy, J. P. and Brudvig, G. W. (2008) Redox reactions of the non-heme iron in photosystem II: an EPR spectroscopic study. Biochemistry 47, 13394–13403

38 Vass, I., Sass, L., Speta, C., Bakou, A., Ghanotakis, D. F. and Petrouleas, V. (1996) UV-B-induced inhibition of photosystem II electron transport studied by EPR and chlorophyll fluorescence. Impairment of donor and acceptor side components. Biochemistry 35, 8964–8973

39 Weisser, J. T., Nilges, M. J., Soer, M. J. and Wilker, J. J. (2006) EPR investigation and spectral simulations of iron-catecholate complexes and iron-peptide models of marine adhesive cross-links. Inorg. Chem. 45, 7736–7747

40 Bruijninckx, P. C., Lutz, M., Spek, A. L., Hagen, W. R., van Koten, G. and Gebbink, R. J. (2007) Iron(III)-catecholate complexes as structural and functional models of the intradiol-cleaving catechol dioxygenases. Inorg. Chem. 46, 8391–8402

41 Hagedorn, P. L., Schmidt, P. P., Andersson, K. K., Hagen, W. R., Flatmark, T. and Martinez, A. (2001) The effect of substrate, dihydrobiopterin, and dopamine on the EPR spectroscopic properties and the midpoint potential of the catalytic iron in recombinant human phenylalanine hydroxylase. J. Biol. Chem. 276, 22850–22856

42 Martinez, A., Andersson, K. K., Haavik, J. and Flatmark, T. (1991) Recombinant human tyrosine hydroxylase isozymes. Construction with iron and inhibitory effect of other metal ions. Eur. J. Biochem. 198, 675–682

43 Hershaw, T. F., Feig, M. and Hausinger, R. P. (2004) Aberrant activity of the DNA repair enzyme AlkB. J. Inorg. Biochem. 98, 856–861

44 Mishina, Y., Chen, L. X. and He, C. (2004) Preparation and characterization of the native iron(ll)-containing DNA repair AlkB protein directly from Escherichia coli. J. Am. Chem. Soc. 126, 16930–16936

45 Ryle, M. J., Liu, A., Muthukumaran, R. B., Ho, R. Y., Koehntop, K. D., McCracken, J., Que, J., L. and Hausinger, R. (2002) O2− and α-keto acid-dependent tyrosyl radical formation in TauD, an α-keto acid-dependent non-heme iron dioxygenase. Biochemistry 41, 1854–1862

46 Orville, A. M. and Lipscomb, J. D. (1993) Simultaneous binding of nitric oxide and isotopically labeled substrates or inhibitors by reduced protocatechuate 3,4-dioxygenase. J. Biol. Chem. 268, 8596–8607

47 Han, A. Y., Lee, A. D. and Abu-Omar, M. M. (2006) EPR and UV–vis studies of the nitric oxide adducts of bacterial phenylalanine hydroxylase: effects of cofactor and substrate on the iron environment. Inorg. Chem. 45, 4277–4283

48 Katoh, K., Kuma, K., Toh, H. and Miyata, T. (2005) MAFFT version 5: improvement in accuracy of multiple sequence alignment. Nucleic. Acids Res. 33, 511–518

49 Waterhouse, A. M., Procter, J. B., Martin, D. M., Clamp, M. and Barton, G. J. (2009) Jalview Version 2 – a multiple sequence alignment editor and analysis workbench. Bioinformatics 25, 1189–1191

50 Yu, B., Edstrom, W. C., Benach, J., Hamuro, Y., Weber, P. C., Gibney, B. R. and Hunt, J. F. (2006) Crystal structures of catalytic complexes of the oxidative DNA/RNA repair enzyme AlkB. Nature 439, 879–884