Available online at www.sciencedirect.com
ScienceDirect
Journal of Inorganic Biochemistry 101 (2007) 1043–1048
JOURNAL OF Inorganic Biochemistry
www.elsevier.com/locate/jinorgbio

Replacement of non-heme Fe(II) with Cu(II) in the α-ketoglutarate dependent DNA repair enzyme AlkB: Spectroscopic characterization of the active site

Boris Bleijlevens [a], Tara Shivarattan [a], Barbara Sedgwick [b], Stephen E.J. Rigby [c], Steve J. Matthews [a,*]

[a] *Division of Molecular Biosciences, Faculty of Natural Sciences, Imperial College London, South Kensington Campus, Biochemistry Building, London SW7 2AZ, UK*
[b] *Cancer Research UK London Research Institute, Clare Hall Laboratories, South Mimms, Hertfordshire EN6 3LD, UK*
[c] *School of Biological and Chemical Sciences, Queen Mary, University of London, Mile End Road, London E1 4NS, UK*

Received 31 October 2006; received in revised form 24 March 2007; accepted 28 March 2007
Available online 21 April 2007

Abstract

The bacterial DNA repair enzyme AlkB is an α-ketoglutarate (αKG) dependent non-heme Fe(II) containing dioxygenase. Here we describe, for the first time, the preparation of a Cu(II)-reconstituted form of AlkB in various complexes. Spectroscopic characterization showed correct AlkB folding upon incorporation of Cu(II) in the active site. The Cu site was classified as a type 2 site by EPR spectroscopy. The accessibility of the active site metal was studied using imidazole as a probe. Although addition of imidazole did not change the EPR spectrum of the AlkB–Cu–αKG complex, the spectrum of the AlkB–Cu–succinate complex clearly changed, indicating binding of imidazole at the Cu site. Binding of substrate (methylated DNA) to the AlkB–Cu–αKG complex did not induce changes in the EPR spectrum, demonstrating that the substrate does not bind in the immediate vicinity of the metal centre. This work provides a basis for advanced EPR approaches aimed at studying the interactions and dynamics of AlkB complexes in solution.

Keywords: AlkB; Non-heme iron; Dioxygenase; Copper; EPR

1. Introduction

The class of α-ketoglutarate (αKG) dependent non-heme Fe(II) containing dioxygenases is known for its catalysis of a wide range of reactions. One relatively new member of this class is the DNA repair enzyme AlkB from *Escherichia coli*. Computational protein-fold analysis showed that AlkB contains the 2-His-1-carboxylate triad characteristic for this class of enzymes [1]. AlkB is functional in the demethylation of methylated bases in DNA [2,3] and RNA [4]. A recently published crystal structure of AlkB revealed a double-stranded β-helical (DSBH) core typical of αKG dependent dioxygenases [5]. The Fe(II) ion in the active site is in near-octahedral symmetry and is coordinated to the protein through His131, Asp133 and His187. Under anaerobic conditions the metal centre is further ligated by two oxygen atoms from αKG and one water molecule (Fig. 1) [5]. Overall, the X-ray structure of AlkB binding the co-product succinate is similar, except for a change in ligand coordination of the metal ion. One of the two oxygen ligands provided by αKG in the holo structure is replaced by water, thus preserving the active site geometry. In addition, AlkB–αKG–complexes binding either cobalt or manganese instead of iron are structurally identical to the native enzyme [5]. No crystal structure was elucidated for AlkB binding Cu(II). Such a complex would allow structural characterization of the active site via solution-state spectroscopy, an approach successfully applied

* Corresponding author. Fax: +44 207 594 3057.
E-mail address: s.j.matthews@imperial.ac.uk (S.J. Matthews).

0162-0134/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.jinorgbio.2007.03.018

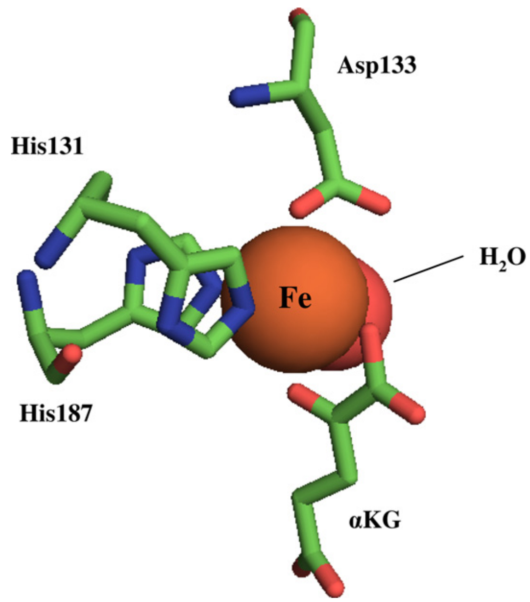

Fig. 1. Representation of the AlkB Fe(II) active site in the presence of the co-substrate α-ketoglutarate (PDB 2FD8). The protein crystal was grown under anaerobic conditions in the presence of the methylated trinucleotide T-1meA-T [5].

to other non-heme iron containing dioxygenases [6–9]. The experiments described in the work presented here were designed to characterize the AlkB active site using EPR spectroscopy. Previous studies on non-heme dioxygenases have shown that the active site iron is in the high-spin ferrous state [10]. High-spin ferrous sites (Fe(II), $3d^6$, $S = 2$) are notoriously difficult to monitor spectroscopically. Although EPR spectroscopy is potentially very powerful in providing structural information about active site metals, high-spin ferrous sites cannot be studied with the standard (perpendicular) form of this technique. To make this system amenable for EPR spectroscopy we removed the Fe(II) ion immediately after purification to substitute it with Cu(II). A stable, fully-structured AlkB–Cu–αKG complex was successfully prepared as adjudged by NMR and near-UV CD spectroscopy. Replacing the Fe(II) ion with Co(II) resulted in a similarly well-folded protein. EPR spectroscopy on Cu(II)-substituted AlkB confirmed binding of Cu(II) to the active site in a geometry similar to a type 2 Cu site. We have studied the metal site in the presence of substrate (methylated DNA), co-substrate (αKG), and co-product (succinate) and used imidazole binding to Cu(II) to probe the ligand coordination of the metal site.

2. Materials and methods

2.1. Protein purification and Cu-reconstitution

Recombinant C-terminally His_6-tagged AlkB was expressed as soluble protein in *E. coli* BL21 (DE3) bearing the recombinant plasmid pET21b [11]. Transformed cells were grown on plates containing 50 μg/ml carbenicillin. One litre of LB was inoculated with an overnight culture and incubated at 37 °C until an OD_{600} of 0.8 was reached. Protein expression was induced by addition of 0.4 mM IPTG (isopropylthiogalactopyranoside) to the culture and incubation at 37 °C for a further 3–4 h. Cells were harvested and resuspended in binding buffer (20 mM Tris–HCl (pH 8.0), 5 mM NaCl, 5 mM imidazole). Following lysis through sonication, the AlkB containing supernatant was loaded onto a Ni-NTA column (Qiagen) equilibrated with binding buffer. Two subsequent washes (with 25 mM and 50 mM imidazole, respectively) were carried out prior to elution in 150 mM imidazole. A chelex-100 (BioRad) column was used to remove bound metal from the protein and obtain the apo protein. Purified apoAlkB was rebuffered to 20 mM Tris–HCl pH 7.6. The purity of the protein was checked by the presence of a single band on SDS-PAGE. Protein yields were ~15 mg/l of bacterial culture.

AlkB was reconstituted with Co(II) or Cu(II) by the addition of aliquots of a $CoCl_2$ or $CuSO_4$ stock solution (2–5 mM) to a mixture of metal-free AlkB (~50–100 μM) and excess αKG (1 mM). Immediate precipitation occurred when excess metal was added to protein, therefore a metal-to-protein ratio of 0.8 to 1 was used for reconstitution.

2.2. Near-UV circular dichroism spectroscopy

Near-UV CD spectra were recorded from 240 to 380 nm to probe tertiary structure of AlkB after reconstitution with metal. Spectra were recorded on a Chirascan CD spectrometer (Applied Photophysics). Samples with protein concentrations of 1 mg/ml (~40 μM) were measured in a SUPRASIL quartz cuvette (QS, Hellma) with a path length of 4 mm. Data were collected for 2 s per data point at 0.5 nm step size with a spectral bandwidth of 1 nm. Presented data are averages of three scans. Because of the instability of AlkB, samples were cooled to 10 °C during recording. Spectra were corrected for background signal from Tris–HCl buffer (end concentration 30 mM); signals from other components were negligible in the near-UV region.

2.3. 1D ^1H NMR spectroscopy

NMR data were recorded on a Bruker Avance 500 MHz spectrometer equipped with a cryoprobe. 1D ^1H NMR spectra were recorded on protein samples (~100 μM) in 20 mM Tris–HCl buffer (pH 7.6). The presented spectrum is an average of 128 scans.

2.4. Electron paramagnetic resonance spectroscopy

EPR spectra were recorded of Cu(II)-reconstituted AlkB (~100 μM) and the effects of the binding of substrate (methylated DNA, 220 μM), product (unmethylated DNA, 234 μM), co-substrate (αKG, 1 mM) and co-product (succinate, 1 mM) were studied. Imidazole (10 mM)