ARTICLE IN PRESS

Biochemical and Biophysical Research Communications xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

The Fe-heme structure of met-indoleamine 2,3-dioxygenase-2 determined by X-ray absorption fine structure

Jade B. Aitken [a,b,c], Christopher J.D. Austin [a,d], Nicholas H. Hunt [d], Helen J. Ball [d], Peter A. Lay [a,*]

[a] School of Chemistry, The University of Sydney, NSW 2006, Australia
[b] Australian Synchrotron, Clayton, Victoria 3168, Australia
[c] Institute of Materials Structure Science, KEK, Tsukuba, Ibaraki 305-0801, Japan
[d] Department of Pathology and Bosch Institute, The University of Sydney, Camperdown, NSW 2006, Australia

ARTICLE INFO

Article history:
Received 7 May 2014
Available online xxxx

Keywords:
Indoleamine 2,3-dioxygenase-2 (IDO2)
X-ray absorption fine structure
EXAFS
Heme environment
Mixed-spin species

ABSTRACT

Multiple-scattering (MS) analysis of EXAFS data on met-indoleamine 2,3-dioxygenase-2 (IDO2) and analysis of XANES have provided the first direct structural information about the axial donor ligands of the iron center for this recently discovered protein. At 10 K, it exists in a low-spin bis(His) form with Fe–N_p(av) = 1.97 Å, the Fe–N_{Im} bond lengths of 2.11 Å and 2.05 Å, which is in equilibrium with a high-spin form at room temperature. The bond distances in the low-spin form are consistent with other low-spin hemeproteins, as is the XANES spectrum, which is closer to that of the low-spin met-Lb than that of the high-spin met-Mb. The potential physiological role of this spin equilibrium is discussed.

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1. Introduction

L-Tryptophan (Trp) is the least abundant, essential amino-acid in mammals [1]. The major route for human Trp metabolism (∼90% of all ingested Trp) is the ‘Kynurenine Pathway’ (KP), which leads to the production of biologically active kynurenine metabolites [2], nicotinamide adenine dinucleotide and niacin [3,4]. The first, rate-limiting step of the KP is the oxidative cleavage of the 2,3-double bond of the indole ring of Trp to form *N*-formylkynurenine. Since the 1960s, this reaction was believed to be catalyzed by two heme-containing dioxygenases, the constitutively expressed tryptophan 2,3-dioxygenase (TDO) [5] and the inflammatory cytokine-induced indoleamine 2,3-dioxygenase-1 (IDO1) [6]. More recently it has been found that mammals possess a third dioxygenase enzyme capable of catalysing the oxidative cleavage of Trp, subsequently named indoleamine 2,3-dioxygenase-2 (IDO2) [7–9]. The discovery of a third initiating protein of the KP has attracted considerable research interest due to the established roles of Trp metabolism [10] and TDO/IDO1 dysfunction in numerous pathological conditions, including cancer [11,12] and malaria [13,14].

Phylogenetic analysis suggests that IDO1 arose in mammals from the gene duplication of an ancestral IDO2-like gene approximately 300 million years ago [15]. Interestingly, while modern IDO1 and IDO2 proteins share some amino acid sequence identity (43% for both the human and mouse proteins) [16], both enzymes show little similarity to TDO, indicating functional convergence in Trp metabolism evolution [9]. This evolutionary diversity is reflected in the distinct tissue expression patterns of each enzyme. In mammals, TDO is primarily expressed in the liver [17]. IDO1, in contrast, is found in almost all tissues and non-hepatic organs [18], including the intestine, placenta, lung, endocrine glands and central nervous systems [19–21]. IDO2 is primarily expressed in the kidney, reproductive tract, and liver [7].

IDO1 is a monomeric protein (MW 42 kDa) that is inactive in its native ferric form. IDO1 metabolic activity requires a reductant co-factor in order to bind Trp with high affinity [22]. *In vitro* IDO1 assays generally use a methylene blue/ascorbic acid reduction system, but the physiological reductant is suggested to be cytochrome b_5 [23]. Similarly IDO2 (MW 42 kDa) must be reduced to its ferrous form for enzymatic activity and cytochrome b_5 is the most efficient reductant *in vitro* [16].

Multiple-scattering (MS) analyses of EXAFS have been used for the structural characterizations of met- and deoxy-myoglobin (Mb) [24], as well as the NO adducts of IDO1 [25]. The validity of the EXAFS model used here has been verified for deoxy- and met-Mb by comparison of the EXAFS-derived bond lengths with those found in subsequent very high resolution XRD structures (∼1 Å) [26,27]. The crystal structure of human IDO1 [28] has been used previously to model a likely structure for IDO2 (mouse) [16];

* Corresponding author.
E-mail address: peter.lay@sydney.edu.au (P.A. Lay).

http://dx.doi.org/10.1016/j.bbrc.2014.05.054
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however, the fine details of the active site/heme structure have not yet been determined experimentally. Hence, we report the first structural information on the heme moiety of mammalian IDO2 protein (*i.e.*, mouse met-IDO2) as determined by multiple-scattering analyses of extended X-ray absorption fine structure (EXAFS).

2. Materials and methods

2.1. Protein preparation

Cloning of the mouse IDO2 cDNA into the pDEST-17 for bacterial-expression of N-terminal 6× His tagged proteins has been described previously [16]. Briefly, cultures of transformed KRX *Escherichia coli* cells (Promega) were grown at 37 °C in 1 L of Terrific Broth medium supplemented with carbenicillin (50 mg/mL) until an OD_{600nm} of 0.8 was reached. The temperature was lowered to 20–22 °C until the OD_{600nm} was between 1.0 and 1.5. Then, expression of a heme-containing IDO2 protein was induced overnight by adding *iso*-propyl βD-1-thiogalactopyranoside (1 mM), rhamnose (0.1% w/v) and the heme precursor, δ-aminolevulinic acid hydrochloride (0.5 mM). KRX *E. coli* were collected by centrifugation at 8000*g* for 15 min, resuspended and incubated for 1 h on ice in 25 mM Tris buffer pH 7.4, 150 mM NaCl, 10 mM imidazole, 10 mM $MgCl_2$, lysozyme (1 mg/mL), EDTA-free cocktail inhibitor tablets (2×), DNase (<1 mg) and 1 mM phenylmethylsulfonyl fluoride (PMSF). The suspension was sonicated (Branson Sonifier 3 × 40 W, 30 s pulses) before clearing the supernate by centrifugation at 5000*g* for 20 min.

The supernate (25 mL) obtained from 1 L of culture was applied to a 1 mL Hi-Trap chelating column (Amersham Biosciences) that had been charged with nickel ions and equilibrated with basal buffer (25 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 mM PMSF, 10 mM imidazole). After washing with 18 mL of basal buffer, the protein was eluted by gradually increasing the concentration of imidazole from 10 mM to 300 mM. Fractions with a high A_{406}:A_{280} ratios (*i.e.*, Soret heme absorbance to protein absorbance ratio) were pooled, desalted into 50 mM Tris–HCl pH = 7.4, 40% glycerol and concentrated to 1 mM using an Amicon Ultra 4 mL centrifugal device (Millipore) with a cut-off of 30000 kDa (utilising IDO2 protein preparations from a total of 28 L of bacterial culture). The protein was stored at −80 °C before analysis.

2.2. X-ray absorption spectra (XAS) data collection

The iron K-edge XAS of the met-IDO2 was recorded at SSRL on beamline 7–3 with a harmonic rejection mirror fully tuned. Data were collected as fluorescence spectra, using a Canberra 30-element Ge detector. The samples were held at a temperature of 10 K using an Oxford Instruments continuous-flow liquid helium cryostat. A calibration channel with an Fe foil was used to account for energy shifts in the monochromator [29]. The energy of the incident beam was calibrated with an iron foil, setting the first inflection point to 7111.2 eV, throughout an E_0 of 7130.0 eV was used. During the collection of data, the edge of each XAS spectrum was monitored for photodecomposition; due to photodamage only one scan was collected on each spot (12 scans in total, with two samples).

2.3. XAFS model

The porphyrin fragment was modelled in two-dimensional space [30] and with fourfold symmetry (Fig. S1). A number of models were considered for both adducts with the S_0^2 value set at 0.97, as was determined for met-myoglobin [24]. The models were: (i) a five-coordinate heme protein with the imidazole ring of the histidine as the proximal axial ligand, as described previously [24,25,30]; (ii) with an aqua ligand as the distal ligand; (iii) one with two *N*-bound histidines; and (iv) another with one *N*-bound and one *C*-bound histidines. The *C*-bound model was included as isomerisation between *N*- and *C*-bound imidazoles in complexes of Ru(II) and Ru(III) has previously been reported [31]; it is conceivable that such isomerisation could also be present in low-spin Fe(III) complexes. A complete set of restraints and constraints for these models is contained in the Supporting Information (Table S1) where σ_{res} is analogous to the estimated standard deviation.

MS analyses of XAFS data were performed using the XFIT program, which employed non-linear least-squares fitting of the XAFS spectrum, through the minimization of the sum of the square of the residuals [24,32]. XFIT integrates the FEFF6.01 program in its model fitting calculations, which included multiple-scattering codes [33,34]. The goodness-of-fit parameter (R) and Monte Carlo calculations were performed as reported previously [24,30,35,36]. The rms (root mean square) errors that result from the Monte Carlo analysis were combined with systematic errors to obtain the final conservative error estimate [30]. All background subtraction, splining, and normalization procedures were performed using XFIT as reported previously [24,30].

A window from 0 to 12 $Å^{-1}$ with a cosine edge function was applied to the XAFS data. The Fourier transform had a window applied from 0.5 to 4.5 Å. This window filtered the majority of the atomic XAFS (between 0 and ∼1.5 Å).

2.3.1. Determinacy

The number of parameters being fitted, *p*, compared to the number of independent information data points (independent points in the EXAFS plus the number of independent structural parameters), N_i, was calculated to give the degree of determinacy N_i/p. If this ratio is <1, then the model is considered to be underdetermined and a unique fit is not possible. In all cases, the ratio was >1 and, hence, the models were overdetermined. The value of N_i is given by [37]: $N_i = 2(\Delta r)(\Delta k)/\pi + \sum[D(N-2)+1]$; where D is the number of dimensions in which the refinement takes place (two for the planar porphyrin and three for the free rotation of the axial groups) and N is the number of atoms in the unit. These calculations are combined to give the determinacy values reported in the tables.

2.3.2. Goodness-of-fit (Residual)

The method of determining the goodness of fit was through an *R*-value, where *R* is given by: $R = (\chi^2/\chi^2_{calculated=0})^{1/2}$ where χ^2 is the quantity that was minimized during the refinement and $\chi^2_{calculated=0}$ is the value of χ^2 when the calculated XAFS is uniformly 0 [33]. Residual *R* values of ⩽20% were considered reasonable [35].

2.3.3. Monte-Carlo error analysis

Monte Carlo analyses were conducted to estimate the rms deviations in final parameters arising from the noise in the data. Two consecutive sets of 16 × 16 Monte-Carlo cycles were calculated and the random (statistical) errors due to noise in the data were estimated by Monte-Carlo calculations, and the systematic errors were assigned a conservative value of 0.02 Å.

3. Results

The XANES edge energy of met-IDO2 was at a similar energy to that of met-Mb [24] and met-leghemoglobin (Lb) [38], 7126.5 eV (Fig. 1). However, the shape of the met-IDO2 spectra was similar to that of low-spin met-Lb rather than high-spin met-Mb. The position and structure of the edge spectrum were sensitive indicators of both the local coordination environment and the photodecom-

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