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Mutation of cysteine residues alters the heme-binding pocket of indoleamine 2,3-dioxygenase-1

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ABSTRACT

The hemoprotein indoleamine 2,3-dioxygenase-1 (IDO1) is the first and rate-limiting enzyme in mammalian tryptophan metabolism. Interest in IDO1 continues to grow, due to the ever expanding influence IDO1 plays in the immune response. This study examined the contribution of all individual cysteine residues towards the overall catalytic properties and stability of recombinant human IDO1 *via* mutagenesis studies using a range of biochemical and spectroscopic techniques, including *in vitro* kinetic assessment, secondary structure identification *via* circular dichroism spectroscopy and thermal stability assessment. Upon mutation of cysteine residues we observed changes in secondary structure (principally, shifting from α-helix/β-sheet features to random coil structures) that produced out of plane heme torsion and puckering, changes to thermal stability (including gains in stability for one mutant protein) and differences in enzymatic activity (such as, increased ability to convert non-natural substrates, *e.g.*D-tryptophan) from wild type IDO1 enzyme.

1. Introduction

The haemoprotein indoleamine 2,3-dioxygenase-1 (IDO1; EC 1.13.11.52) is an immunoregulatory enzyme responsible for the oxidative cleavage of L-tryptophan to *N*′-formylkynurenine as the first step of the Kynurenine Pathway; the major metabolic pathway for tryptophan breakdown (Fig. 1; Left). Increased expression of IDO1, principally by IFN-γ release due to inflammation resulting from infection and/or disease, elicits both innate and adaptive immune responses that can have both beneficial and detrimental consequences. For example, essential amino acid removal such as tryptophan depletion by increased expression of IDO1 is an ancient, efficient and innate method of slowing the growth of infectious agents that rely on endogenous tryptophan stores for continued metabolic activity [1]. Furthermore, the low tryptophan cellular environment changes adaptive immune mechanisms, altering modulatory signals to/from T-lymphocytes, resulting in both G1-cell cycle arrest and apoptosis. This in turn causes host immunosuppression and is a major mechanism of both tumour immune escape [2] and prevention of maternal foetal rejection [3,4]. In addition, IDO1 activity affects the production of down-stream metabolites of tryptophan (e.g. 3-hydroxykynurenine and quinolinic acid), which have been shown to play roles in inflammatory disease symptomology, particularly in the CNS [5,6]. Consequently, IDO1 has received significant research attention as a potential therapeutic target in disease states where the immune system is in a state of dysregulation, for example, ovarian cancer [7]. A greater understanding of how the structural components of human IDO1 interact (*vide infra*), and how alteration of these interactions govern both function and stability, may aid both future drug development and identification and characterisation of possible deleterious single nucleotide polymorphisms [8].

Cysteine residues in proteins are important sites for metal coordination, catalysis and structure stabilisation. Containing a reactive sulfhydryl (–SH) group, the cysteine moiety can act as a nucleophile and forms thiol radicals, making it possible to form both intra- and intermolecular covalent bonds. However, the ease of formation of such covalent bonds depends on the overall redox potential, the spatial environment and pH of the protein and surrounding environment. In IDO1, cysteines are well distributed, mostly in helices. Three cysteine residues are found in the small domain (which contains nine α-helices and two β-sheets) and five in the large domain (comprising 15 α-helices and the catalytic pocket – Fig. 1; Right). IDO1 cysteines do not form disulfide bridges or co-ordinate to the heme iron so their role in the maintenance of activity/structure remains largely unknown. We report here on the contribution of all the individual cysteine residues towards the

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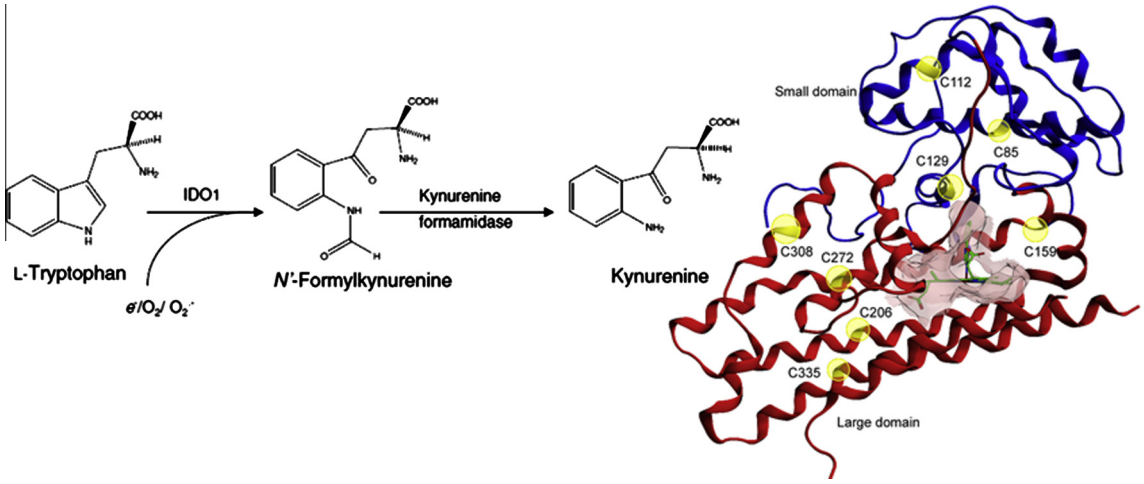

Fig. 1. (Left): initial steps of the Kynurenine pathway. The heme containing enzyme indoleamine 2,3-dioxygenase (IDO) is capable of catalysing this reaction. The cleavage product, *N*′-formylkynurenine, is then hydrolysed by kynurenine formamidase, or spontaneously, to form kynurenine. (Right) Crystal structure of human IDO-1 showing positions of all 8 cysteine residues and the active site (PDB: 2D0T, [16]). Active site – pink; heme and co-crystalised phenylimidazole – green. The large and small domains are coloured red and blue, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

overall catalytic properties, secondary structure, heme environment and stability of human IDO1.

2. Materials and methods

2.1. Materials

All chemicals were of analytical grade unless otherwise specified. A Hi-Trap chelating column and Microspin Sephadex G-25 columns were obtained from Amersham Biosciences. Sephadex G25 (NAP 10) columns were purchased from Pharmacia Biotech. Amicon Ultra 4 mL centrifugal devices were obtained from Millipore. A Superdex 75 PC 3.2/30 column was purchased from Pharmacia LKB Biotechnology. δ-Aminolevulinic acid (ALA), ampicillin, ascorbic acid, catalase (bovine), imidazole, isopropyl-β-D-thiogalactopyranoside (IPTG), L-kynurenine, kanamycin, lysozyme, *p*-dimethylaminobenzaldehyde (*p*-DMAB), phenylmethylsulfonylfluoride (PMSF) and L-tryptophan were obtained from Sigma–Aldrich. Bovine serum albumin (BSA) (Fraction V) was obtained from Amersham. Coomassie blue R250 was purchased from Bio-Rad. DNase and EDTA-free cocktail inhibitor tablets were products of Roche.

2.2. Site-directed mutagenesis of the IDO1 expression plasmid pQE9-IDO1

Plasmid DNA was isolated using a GenElute Plasmid Miniprep Kit (Sigma), according to the manufacturer's instructions. Site-directed mutagenesis of pQE9-IDO1 was undertaken using the QuikChange® site-directed mutagenesis kit (Stratagene) per the manufacturer's instructions. All primers were synthesised by Sigma–Genosys (Castle Hill, Australia). Primers used for site-directed mutagenesis and sequencing are outlined in Supplementary Data. Dye terminator DNA sequence analysis was performed using an ABI-PRISM 377 DNA sequencer (Applied Biosystems) to confirm mutations.

2.3. Bacterial Strain

The *Escherichia coli* strain (EC538, pREP4) used for expression studies has been previously used for the recombinant expression of human IDO1 [9]. Briefly, a single colony of *E. coli* (EC538) cells containing plasmids pQE9-IDO and pREP4 was inoculated in 100 mL LB medium and cultured overnight. The 100 mL culture was added to 900 mL of the same medium and incubated at 30 °C to an optical density of 0.6 at 600 nm. IPTG (100 mM), ALA (500 mM), and PMSF (1 M) were then added at final concentrations of 0.1, 0.5, and 1 mM, respectively. Each culture was incubated for a further 3 h. Cells were collected as a pellet by centrifugation at 5000*g* for 20 min at 4 °C. The pellet was suspended in 20 mL ice-cold (Dulbecco's) phosphate–buffered saline (PBS) containing 1 mM PMSF and centrifuged at 3000*g* for 15 min at 4 °C. The bacterial pellet was stored at −20 °C.

2.4. Purification of wild type IDO1 and mutant IDO1 proteins

One litre pellets of bacterial culture, obtained according to the method described above, was suspended in 25 mM tris(hydroxymethyl)methylamine (Tris) buffer at pH 7.4, containing 150 mM NaCl, 10 mM imidazole, 10 mM $MgCl_2$ and 1 mM PMSF. The suspension was then centrifuged at 5000*g* for 20 min at 4 °C and the supernatant discarded to remove any residual PBS storage buffer.

The newly washed pellets were resuspended in 20 mL of ice-cold buffer as outlined above, with the addition of EDTA free-cocktail inhibitor tablets (×2) and DNase (<1 mg). The suspension was French pressed three times at 10,000 p.s.i. and centrifuged at 5000*g* for 20 min to obtain a clear supernatant and pellet. The clear supernatant (20 mL) was then applied to a 1 mL Hi-Trap chelating column charged with nickel ions; equilibrated with the basal buffer [Tris 25 mM pH 7.4; 150 mM NaCl; 1 mM PMSF] containing 10 mM imidazole. After washing with 18 mL of this buffer, IDO1 (wild type or mutant) protein was eluted on a stepwise gradient incorporating washings at imidazole concentrations of 30, 40, 50, 65, 80 mM, and elution at 190 mM.

The protein collected at the elution step was then buffer-exchanged into 50 mM Tris pH 7.4 using a Sephadex G25 (NAP 10) column. The desalted fractions were pooled and concentrated to a volume of 50 μL using an Amicon Ultra 4 mL centrifugal device with a 30,000 Da molecular weight cut-off. The concentrated fraction was then applied to a Superdex 75 PC 3.2/30 column according to the manufacturer's instructions, after equilibration with 50 mM Tris pH 7.4. Fractions were collected in 75 μL aliquots at a flow rate of 60 μL/min over 3.5 mL. The fractions with the highest 406:280 nm absorbance were pooled for analysis.

2.5. Analysis of wild type IDO1 and mutant IDO1 proteins

Protein concentration was determined with Bio-Rad dye reagent using BSA (0–1 mg/mL) as the standard. The coloured prod-