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ORIGINAL ARTICLE

Structural basis of fumarate hydratase deficiency

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Abstract Fumarate hydratase catalyzes the stereospecific hydration across the olefinic double bond in fumarate leading to L-malate. The enzyme is expressed in mitochondrial and cytosolic compartments, and participates in the Krebs cycle in mitochondria, as well as in regulation of cytosolic fumarate levels. Fumarate hydratase deficiency is an autosomal recessive trait presenting as metabolic disorder with severe encephalopathy, seizures and poor neurological outcome. Heterozygous mutations are associated with a predisposition to cutaneous and uterine leiomyomas and to renal cancer. The crystal structure of human fumarate hydratase shows that mutations can be grouped into two distinct classes either affecting structural integrity of the core enzyme architecture, or are localized around the enzyme active site.

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References to electronic databases: OMIM: 606812 150800, 605839, 136850; EC 4.2.1.2; Gene symbol: *FH*; GenBank: 19743875; URL to the interactive version of the article: http://www.ssiem.org/resources/structures/FH/; PDB code: 3E04

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An interactive version of this manuscript (which may contain additional mutations appended after acceptance of this manuscript) may be found on the SSIEM website at:

http://www.ssiem.org/resources/structures/FH.

Abbreviations

FH	Fumarate hydratase
FHD	Fumarate hydratase deficiency
MCUL1	Multiple cutaneous and uterine leiomyomata
HLRC	Hereditary leiomyomatosis and renal cancer syndrome

Introduction

Fumarate hydratase (FH) and succinate dehydrogenase are two integral enzyme components of the Krebs cycle, and besides their essential role in the TCA cycle, can act as tumour suppressors (King et al. 2006). The *FH* gene codes for fumarate hydratase (or fumarase; EC 4.2.1.2), which catalyzes the stereospecific, reversible hydratation of fumarate to *L*-malate. The *FH* gene localized at 1q42.1 codes for differentially processed, but sequence-wise identical cytosolic and mitochondrial forms. Whereas the mitochondrial enzyme is part of the TCA cycle, the cytosolic form is thought to utilize fumarate derived from different sources. Deficiency in FH activity causes an impaired energy production by interrupting the flow of metabolites through the Krebs cycle. Accumulation of fumarate is thought to competitively inhibit 2-oxo-glutarate dependent dioxygenases that regulate hypoxia inducible factor (HIF), thus activating oncogenic hypoxia pathways (Ratcliffe, 2007).

Due to their essential role in energy production, enzyme deficiencies result in early onset of severe encephalopathy (Kerrigan et al. 2000). Accordingly, autosomal recessive fumarate hydratase deficiency (FHD) caused by mutations in the FH gene results in fumaric aciduria, and common clinical

Table 1 X-ray data collection and refinement statistics

Data collection	
Space group	$P3_221$
a, b, c (Å)	188.5, 188.5, 114.6
γ	120°
Wavelength (Å)	1.000
Resolution (Å)*	25.0 – 1.95 (2.06 – 1.95)
R_{merge} (%)*	0.141 (0.732)
I/σI*	9.7 (2.0)
Completeness (%)*	99.3 (96.8)
Redundancy*	6.2 (5.0)
Refinement	
Resolution (Å)	41.27 – 1.90
No. reflections	168629
R_{work}/R_{free} (%)	19.7/24.4
No. atoms	
Protein	13160
Ligand/ion	12
Water	655
B-factors (Å^2)	
Main-chain	24.88
Side-chain and water	25.91
RMS deviations	
Bond lengths (Å)	0.010
Bond angles (°)	1.201
PDB code	3E04 (doi:10.2210/pdb3e04/pdb)

* Numbers in parentheses represent data in the highest resolution shell.

features observed are hypotonia, failure to thrive, severe psychomotor retardation, seizures, facial dysmorphism and brain malformations. Interestingly, whereas homozygous FH mutations predispose to fumaric aciduria, several heterozygous FH mutations are known to be involved in the autosomal dominant syndrome of multiple cutaneous and uterine leiomyomata (MCUL1) (Tomlinson et al. 2002). Affected individuals develop benign smooth muscle tumours of the skin, and females develop fibroids of the uterus. When co-existing with an aggressive form of renal cell carcinoma (papillary renal type II cancer or renal collecting duct cancer) it is also known as hereditary leiomyomatosis and renal cancer (HLRCC) syndrome. In MCUL1/HLRCC germline mutations in FH are detected in the majority of the screened cases. To date, 107 variants have been described, of which 93 are thought to be pathogenic (Bayley et al. 2008). The most common types are missense mutations (57%), followed by frameshift and nonsense mutations (27%), as well as diverse deletions, insertions and duplications.

Here we present the crystal structure of human fumarase at 1.95 Å resolution and summarize structure-activity correlation between observed mutations and clinical phenotypes.

Materials and methods

Expression, purification & crystallization

DNA fragment encoding the fumerase domain of human FH (aa 44-510; GenBank entry 19743875) was subcloned into

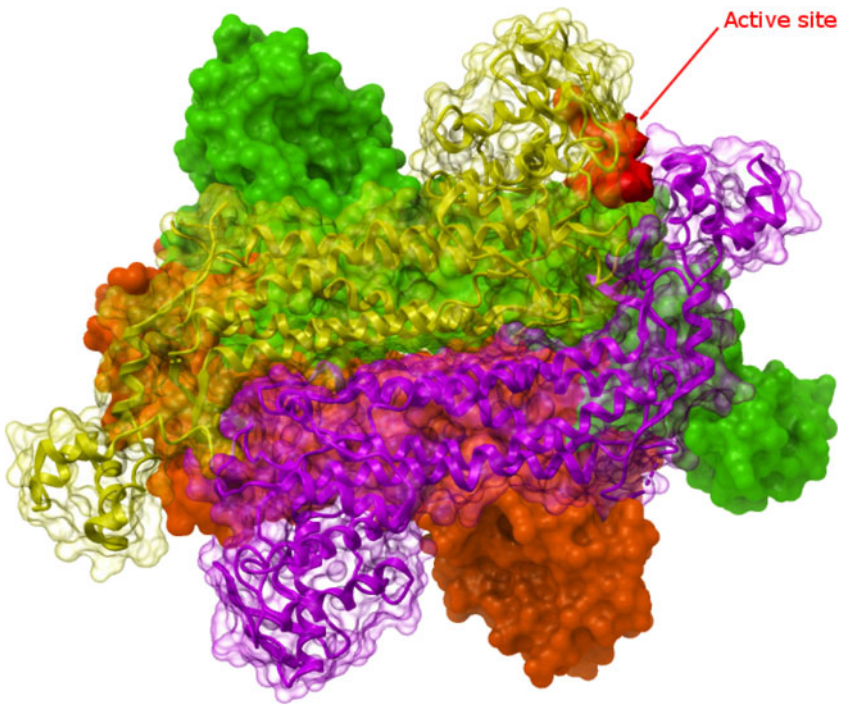

Fig. 1 Ribbon/surface diagram of human fumarate hydratase illustrating the tetrameric assembly of class II fumarases. Molecular surface representation is used to convey the overall shape of each monomer as well as the tetrameric assembly. Each monomer has been coloured distinctively, to facilitate visualization. Two monomers are represented using semi-transparent surfaces, to highlight the fold (represented as ribbons). One of the active sites is highlighted in red, showing contribution of three distinct subunits. The figures were generated using the program ICM (www.molsoft.com)

Table 2 Mutations observed in the human fumarase gene and association to disease. Abbreviations: CL: cutaneous leiomyoma; FHD: fumarate hydratase deficiency; HLRCC: hereditary leiomyomatosis and renal cell cancer; LCT: Leydig cell tumors; MCUL: multiple cutaneous and uterine leiomyomata; OMC: ovarian mucinous cystadenoma; RCC: renal cell carcinoma; STS: soft tissue sarcoma; UL: uterine leiomyomas; ULMS: uterine leiomyosarcoma

#	Mutation site	Mutated residue	Protein change	DNA change	Exon	Conservation	Localization	Reference	Phenotype
1	Arg51	Glu	R51E	c.152 G>A	2	Conserved	Surface	(Kiuru et al. 2002)	STS
2	Arg101	Pro	R101P	c.302 G>C	3	Semi-conserved	Surface	(Chan et al. 2005), (Heinritz et al. 2008)	HLRCC
3	Asn107	Thr	N107T	c.320A>C	3	Conserved	Active site	(Tomlinson et al. 2002), (Alam et al. 2005a), (Carvajal-Carmona et al. 2006)	MCUL, LCT
4	Ala117	Pro	A117P	c.349 G>C	3	Semi-conserved	Near active site	(Tomlinson et al. 2002)	MCUL
5	Leu132	Ser	L132S	c.395 T>C	4	Semi-conserved	Surface	(Wei et al. 2006)	HLRCC, reduced FH activity
6	His135	Arg	H135R	c.404A>G	4	Semi-conserved	Surface	(Chuang et al. 2005)	MCUL
7	Gln142	Lys	Q142K	c.424 C>A	4	Conserved	Near active site	(Badeloe et al. 2006)	MCUL
8	Ser158	Ile	S158I	c.473 G>T	4	Semi-conserved	Near active site	(Martinez-Mir et al. 2003)	MCUL
9	Arg160	Gly	R160G	c.478A>G	4	Conserved	Surface	(Wei et al. 2006)	MUCL, reduced FH activity
10	Pro174	Arg	P174R	c.521 C>G	4	Not conserved	Surface	(Alam et al. 2005b), (Zeng et al. 2006), (Pollard et al. 2005)	FHD
11	His180	Arg	H180R	c.539A>G	4	Semi-conserved	Active site	(Tomlinson et al. 2002), (Alam et al. 2005b)	MUCL
12	Gln185	Arg	Q185R	c.554A>G	4	Conserved	Active site	(Tomlinson et al. 2002)	MCUL
13	Ser187	Leu	S187L	c.560C>T	5	Conserved	Active site	(Toro et al. 2003)	MCUL
14	Asn188	Ser	N188S	c.563A>G	5	Conserved	Active site	(Toro et al. 2003)	MCUL
15	Pro192	Leu	P192L	c.575A>G	5	Conserved	In core helice	(Chuang et al. 2005)	MCUL
16	Met195	Thr	M195T	c.584 T>C	5	Conserved	In core helice	(Toro et al. 2003)	MCUL
17	His196	Arg	H196R	c.587A>G	5	Conserved	In core helice	(Kiuru et al. 2002), (Lehtonen et al. 2004)	RCC, ULMS
18	Ile229	Thr	I229T	c.686 T>C	5	Not conserved	Surface	(Alam et al. 2005b)	MCUL
19	Lys230	Arg	K230R	c.689A>G	5	Conserved	Subunit stabilization	(Tomlinson et al. 2002), (Coughlin et al. 1998), (Manning et al. 2000)	FHD
20	Arg233	Cys	R233C	c.697 C>T	5	Conserved	Active site	(Rustin et al. 1997), (Chuang et al. 2005), (Wei et al. 2006)	FHD, HLRCC, MCUL
21	Arg233	His	R233H	c.698 G>A	5	Conserved	Active site	(Tomlinson et al. 2002), (Alam et al. 2005b), (Wei et al. 2006), (Chuang et al. 2005), (Toro et al. 2003)	HLRCC, MCUL
22	Arg233	Leu	R233L	c.698 G>T	5	Conserved	Active site	(Chuang et al. 2005), (Toro et al. 2003)	MCUL
23	Ala239	Thr	A239T	c.715 G>A	5	Conserved	Near active site	(Lehtonen et al. 2004)	UL
24	Ala274	Thr	A274T	c.820 G>A	6	Not conserved	Active site	(Ylisaukko-oja et al. 2006)	OMC
25	Gly282	Val	G282V	c.845 G>T	6	Conserved	Active site	(Tomlinson et al. 2002), (Alam et al. 2005b)	MCUL
26	Ala308	Thr	A308T	c.922 G>A	7	Conserved	Surface	(Coughlin et al. 1998)	FHD
27	Asn310	Tyr	N310Y	c.928A>T	7	Conserved	Surface	(Alam et al. 2005b)	MCUL
28	Phe312	Cys	F312C	c.935 T>G	7	Conserved	Surface	(Coughlin et al. 1998)	FHD
29	His318	Tyr	H318Y	c.952 C>T	7	Semi-conserved	In core helice	(Toro et al. 2003), (Martinez-Mir et al. 2003)	HLRCC
30	His318	Leu	H318L	c.953A>T	7	Semi-conserved	In core helice	(Deschauer et al. 2006)	FHD
31	Val322	Asp	V322D	c.964 T>A	7	Conserved	In core helice (interaction with 1 other monomer)	(Toro et al. 2003)	MCUL
32	Thr330	Pro	T330P	c.988A>C	7	Semi-conserved	In core helice (interaction with 1 other monomer)	(Chuang et al. 2005)	MCUL
33	Cys333	Tyr	C333Y	c.998 G>A	7	Semi-conserved	In core helice (interaction with 1 other monomer)		MCUL
34	Ser334	Arg	S334R	c.1002 T>G	7	Conserved	In core helice (interaction with 1 other monomer)	(Badeloe et al. 2006)	CL
35	Leu335	Pro	L335P	c.1004 T>C	7	Conserved	In core helice	(Toro et al. 2003)	MCUL

Table 2 (continued)

#	Mutation site	Mutated residue	Protein change	DNA change	Exon	Conservation	Localization	Reference	Phenotype
36	Asn340	Lys	N340K	c.1020 T>A	7	Semi-conserved	In core helice	(Toro et al. 2003), (Wei et al. 2006)	MCUL
37	Glu355	Lys	E355K	c.1063 G>A	7	Conserved	Subunit stabilization	(Alam et al. 2005b)	MCUL
38	Asn361	Lys	N361K	c.1083 T>A	7	Conserved	Active site	(Alam et al. 2005b)	HLRCC-CDC
39	Glu362	Gln	E362Q	c.1084 G>C	7	Conserved	Active site	(Bourgeron et al. 1994)	FHD
40	Ser365	Gly	S365G	c.1093 G>A	7	Conserved	Active site	(Toro et al. 2003), (Wei et al. 2006)	MCUL
41	Ser366	Asn	S366N	c.1097 G>A	7	Conserved	Active site (but out)	(Toro et al. 2003), (Alam et al. 2005b)	MCUL
42	Met368	Thr	M368T	c.1103 T>C	7	Conserved	Active site	(Badeloe et al. 2006)	MCUL
43	Pro369	Ser	P369S	c.1105 C>T	7	Conserved	Active site (but out)	(Maradin et al. 2006)	FHD
44	Asn373	Ser	N373S	c.1118A>G	8	Conserved	Active site	(Lehtonen et al. 2004)	HLRCC/clear cell RCC
45	Gln376	Pro	Q376P	c.1127A>C	8	Conserved	In core helice (interaction with 1 other monomer)	(Zeman et al. 2000), (Remes et al. 2004), (Phillips et al. 2006)	FHD
46	Ala385	Asp	A385D	c.1154 C>A	8	Not conserved	In core helice (interaction with 2 other monomers)	(Wei et al. 2006)	MCUL
47	Val394	Leu	V394L	c.1180 G>C	8	Not conserved	In core helice	(Martinez-Mir et al. 2003)	MCUL
48	Gly397	Arg	G397R	c.1189 G>A	8	Semi- conserved	In core helice	(Alam et al. 2005b)	MCUL
49	His402	Cys	H402C	c.1207 C>T	8	Conserved	In core helice turn (interaction with 2 other monomers)	(Phillips et al. 2006)	FHD
50	Ser419	Pro	S419P	c.1255 T>C	9	Conserved	In core helice	(Wei et al. 2006)	HLRCC
51	Asp425	Val	D425V	c.1274A>T	9	Conserved	In core helice (interaction with 1 other monomer)	(Coughlin et al. 1998)	FHD
52	Gln439	Pro	Q439P	c.1316A>C	9	Not conserved	Surface	(Wei et al. 2006)	HLRCC
53	Met454	Ile	M454I	c.1362 G>A	9	Conserved	Subunit interaction	(Carvajal-Carmona et al. 2006)	LCT
54	Tyr465	Cys	Y465C	c.1394A>G	10	Semi- conserved	Surface	(Toro et al. 2003)	MCUL
55	Leu507	Pro	L507P	c.1520 T>C	10	Semi- conserved	Surface near opening active site	(Alam et al. 2005b)	MCUL

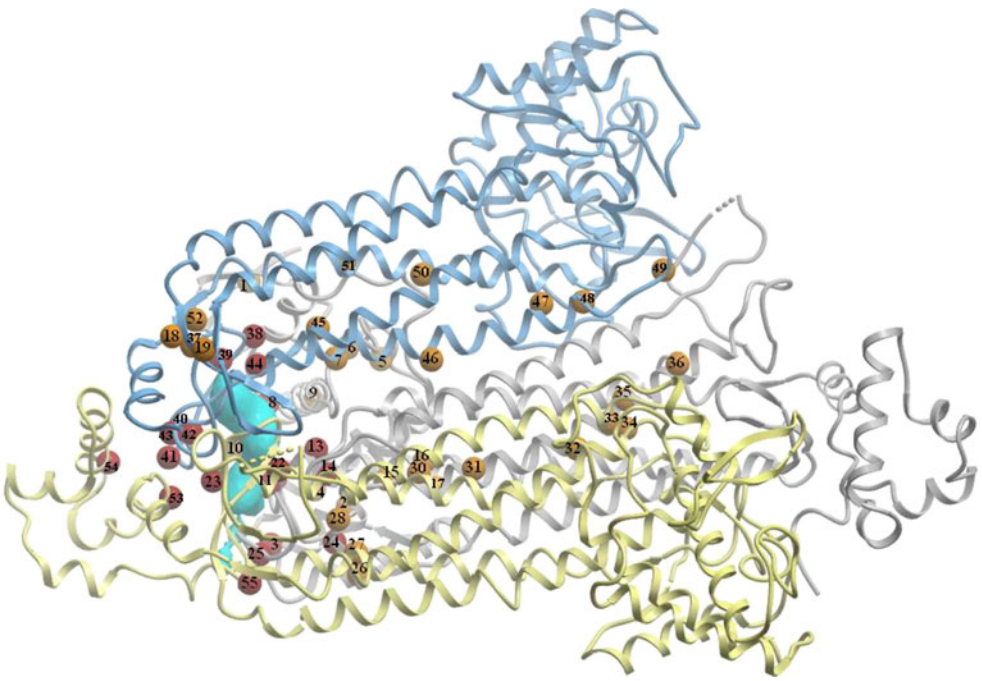

Fig. 2 Clustering of human fumarase missense mutations observed in FHD, MCUL1 and HLRCC. The active site is highlighted in cyan. Positions of amino acid mutations are indicated as small spheres and numbered according to Table 2. The positions around the active site are indicated in red, mutations affecting inter- or intrasubunit interactions are indicated in dark yellow. For clarity, one monomeric subunit is omitted

pNIC28-Bsa4 vector incorporating an N-terminal His_6-tag. The plasmid was transformed into BL21(DE3)-pRARE, cultured in Terrific Broth at 37°C, and induced with 0.1 mM IPTG overnight at 18°C. Cells were homogenized in lysis buffer (50 mM K-phosphate pH 7.5, 500 mM NaCl, 1 mM TCEP), centrifuged to remove cell debris, and the supernatant was purified by Nickel affinity (HisTrap Crude FF) and size exclusion (HiLoad 16/60 Superdex S200) chromatography. Purified protein was concentrated to 12.6 mg/ml and stored in 10 mM HEPES pH 7.5, 150 mM NaCl, 5% (w/v) glycerol and 0.5 mM TCEP at -80°C. Crystals were grown by vapour diffusion at 20°C in sitting drops mixing 150 nl protein and 150 nl reservoir solution containing 20% (w/v) PEG 3350, 0.2 M sodium acetate, 10% (w/v) ethylene glycol and 100 mM Bis-Tris propane pH 7.5. Crystals were cryo-protected in mother liquor containing 25% (w/v) glycerol and flash-frozen in liquid nitrogen.

Data collection & structure determination

Diffraction data to maximum resolution of 1.95 Å were collected on beamline X10A at the Swiss Light Source, and processed using the CCP4 Program suite (CCP4, 1994). FH crystallized in the trigonal space group $P3_221$ (a=180.5 Å, b=180.5 Å, c=114.6 Å, $\alpha=90^o$, $\beta=90^o$, $\gamma=120^o$) with four molecules in the asymmetric unit. The structure of FH was solved by molecular replacement with PHASER (McCoy et al. 2005), using the yeast fumerase structure as search model (PDB code 1YFM). Initial automated model building was performed with ARP/wARP (Perrakis et al. 1999). This is followed by cycles of iterative manual model building using COOT (Emsley & Cowtan 2004) and restrained refinement using REFMAC5 with TLS parameters (Murshudov et al. 1997). The final structure was deposited in the Protein Data Bank (www.rcsb.org) under accession code 3E04 (Table 1).

Results and discussion

Fumarases are divided into two distinct groups. Class I fumarases are iron-dependent iron-sulfur cluster containing, dimeric enzymes, whereas the class II enzymes, including human and other eukaryotic fumarases, are homotetrameric enzymes with a molecular mass of about 200 kDa. Class II fumarases are evolutionarily highly conserved enzymes, e.g. the pairwise identity between *E. coli* and human fumarase is about 60%. Every monomer exhibits a typical tridomain structure, with a central domain involved in subunit interaction, thus forming a typical bundle comprised of 20 α-helices (Fig. 1A). Previous crystallographic analyses have revealed two distinct sites (A and B) in *E. coli* fumarase that can bind carboxylic acids. Site A is formed from three different monomer chains and likely to be the catalytic site, whereas site B is thought to allosterically regulate activity (Rose and Weaver 2004).

A previous study correlated 27 distinct missense mutations to the *E. coli* fumarase structure (Alam 2005b), since then the list of mutations has doubled. To this end, 55 missense mutations in the human fumarase gene are now described. Here we correlate this updated list of mutations to fumarase deficiency, MCUL1 and HLRCC syndrome (Table 2) by using the human fumarase structure. Although not all of these novel mutations have been biochemically characterized, previous results suggest that FH activity is related to HLRCC (Alam 2005a), although other environmental or genetic factors likely play a role in the etiology of the disease. The clustering of mutational "hotspots" suggests enzyme activity relationships to phenotypic appearances. Figure 2 illustrates the clustering of *FH* mutations observed in FHD, MCUL1 and HLRCC. The large majority of mutations are located at evolutionarily highly conserved positions (Table 2) indicating that these mutations likely affect stability and/or activity of the enzyme. Two major clusters of mutations are observed; one is likely to affect structural integrity of the enzyme by interrupting inter or intrasubunit interactions (indicated in yellow in Fig. 2), whereas the other mutations are located around the active site and likely directly affect activity.

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