Biochemical and Biophysical Research Communications xxx (2017) 1–7

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Structural bases of the altered catalytic properties of a pathogenic variant of apoptosis inducing factor

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ARTICLE INFO

Article history:
Received 16 June 2017
Received in revised form
22 June 2017
Accepted 26 June 2017
Available online xxx

Keywords:
Neurodegeneration
Flavoprotein
Protein-ligand interaction
Charge-transfer complex

ABSTRACT

The apoptosis-inducing factor (AIF) is a FAD-containing protein playing critical roles in caspase-independent apoptosis and mitochondrial respiratory chain biogenesis and maintenance. While its lethal role is well known, the details of its mitochondrial function remain elusive. So far, nineteen allelic variants of AIF have been associated to human diseases, mainly affecting the nervous system. A strict correlation is emerging between the degree of impairment of its ability to stabilize the charge-transfer (CT) complex between FAD and NAD^+ and the severity of the resulting pathology. Recently, we demonstrated that the G307E replacement in murine AIF (equivalent to the pathogenic G308E in the human protein) dramatically decreases the rate of CT complex formation through the destabilization of the flavoprotein interaction with NAD(H). To provide further insights into the structural bases of its altered functional properties, here we report the first crystal structure of an AIF pathogenic mutant variant in complex with NAD^+ (murine AIF-$G307E^{CT}$) in comparison with its oxidized form. With respect to wild type AIF, the mutation leads to an altered positioning of NAD^+ adenylate moiety, which slows down CT complex formation. Moreover, the altered balance between the binding of the adenine/nicotinamide portions of the coenzyme determines a large drop in AIF-G307E ability to discriminate between NADH and NADPH.

The apoptosis inducing factor (AIF) [1,2] is a mitochondrial flavoprotein, highly conserved in vertebrates and with homologs in most eukaryotes [2,3]. It is encoded by the *AIFM1* gene on the X chromosome and translated as a precursor apoprotein with an N-terminal mitochondrial localization sequence (MLS) and two nuclear localization signals (NLS) [4]. The mature form of the protein, AIFΔ1-54, is tethered to the intermembrane side of mitochondrial inner membrane *via* its N-terminal segment [1]. As other mitochondrial proteins, AIF has a role in programmed cell death, being the main mediator of caspase-independent apoptosis [1,2]. Apoptotic stimuli can produce the soluble AIFΔ1-102 form, which is released into the cytosol [5,6] and, if translocated into the nucleus, can bind DNA to promote chromatin condensation and large-scale DNA degradation [7]. However, AIF is also critical for the integrity of mitochondria in healthy cells [5,8,9]. Down-regulation of *AIFM1* expression impairs oxidative phosphorylation (OXPHOS), mainly affecting complexes I and III [5,10], suggesting that AIF has a function in respiratory chain biogenesis and/or maintenance [2]. This hypothesis is supported by the recent discovery that AIF is required for the translation-coupled import and activity of CHCHD4 [11], an intermembrane mitochondrial space protein that participates to the oxidative folding of respiratory complexes subunits [12].

Most of the molecular mechanism by which AIF assists mitochondrial structure and functions still remains elusive. However, AIF interaction with NAD(H) seems to be pivotal for the vital functions of the flavoprotein [2,13]. NAD(H)-binding to AIF yields an

Abbreviations: AIF^{CT}, AIF forms in CT complex with NAD^+; AIF^{OX}, AIF forms harboring oxidized FAD; CHCHD4, coiled-coil-helix-coiled-coil-helix domain-containing protein 4; CT, charge transfer; DCIP, 2,6-dichlorophenolindophenol; $FADH^-$, anionic dihydroquinone form of FAD; OXPHOS, oxidative phosphorylation.

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http://dx.doi.org/10.1016/j.bbrc.2017.06.156
0006-291X/

exceptionally oxygen-stable $FADH^{-}$-NAD^{+} charge-transfer complex (AIF^{CT}) that leads to large conformational rearrangements and dimerization of the protein [13–15]. These features led to the hypothesis that AIF could represent a redox and/or NADH sensor, taking part to a novel signal-transduction pathway [2,13]. Moreover, AIF^{CT} formation and protein dimerization are known to prevent its nuclear localization, to weaken its interaction with DNA and to hamper interactions with pro-survival cytoplasmatic partners [13].

OXPHOS defects are typically associated with neurodegeneration [16]. Both the Harlequin mouse, a natural murine strain where AIF level is decreased by 80%, and experimental models of AIF deficiency display OXPHOS defects, mitochondrial alterations and neuron loss [2,9]. To date, nineteen human pathogenic AIF allelic variants have been identified, eleven of which associated with auditory neuropathy spectrum disorders [17], and the others causing neurodegenerative diseases of different degrees of severity [18–25]. Among the latter group, we focused our attention on the G308E replacement, responsible of a rare mitochondrial encephalopathy [19]. We have recently reported the thorough biochemical characterization of murine AIF-G307E, equivalent to human AIF-G308E, demonstrating that this replacement selectively slows down the rate of AIF^{CT} formation [26]. The effect of the amino acid replacement on the reaction between the AIF variant harboring oxidized FAD (AIF-$G307E^{OX}$) and NADH was particularly dramatic, indicating strong destabilization of the initial complex [26]. Our results were fully confirmed by a similar study carried out on the human AIF-G308E variant [27], thus supporting the concept that the mouse homolog is an excellent model of human AIF. While only the crystal structure of the oxidized form of the human AIF-G308E variant has been reported so far [27], we succeeded in determining that of murine AIF-$G307E^{CT}$, the first CT complex of a pathogenic AIF variant, here described in comparison with the respective oxidized form. We also report new relevant details of the effects of the G307E replacement on the catalytic properties of AIF, which prove that the conformational alterations observed in the crystal are maintained in solution. These new findings provide a comprehensive picture of the impact of the G307E replacement on the reactivity of AIF towards nicotinamide ligands.

1. Materials and methods

Expression and purification of AIF forms. Mouse wild type AIFΔ1-101 and its G307E variant were produced and purified as already described [26]. Purified proteins were stored at −80 °C in 50 mM Tris-HCl (pH 7.4), 10% glycerol, and their concentration was determined spectrophotometrically using an ε_{452} of 12.8 $mM^{-1}cm^{-1}$.

Crystallization and crystal data collection. Before crystallization trials, dimeric AIF-$G307E^{CT}$ was generated mixing 250 μM (14 mg/mL) oxidized protein with 2.5 mM NADH for 3 h at 0 °C in 100 mM bis-tris propane (pH 7.4). The complex was then isolated by gel filtration on a Superdex 200 column (GE Healthcare) and concentrated to about 12 mg/mL 1 mM NADH was then added to avoid CT complex reoxidation during crystal growth. Crystals of AIF-$G307E^{OX}$ and AIF-$G307E^{CT}$ were grown at 20 °C in vapour-diffusion set up (Oryx-8 crystallization robot; Douglas Instruments, East Garston, UK) from a 2:1 mixture of protein and reservoir solution (drop volume 0.3 μL). Crystals of AIF-$G307E^{OX}$ were obtained after one day of vapour diffusion against 30% PEG 4000 and 0.2 M sodium acetate (pH 8.5). Crystals of AIF-$G307E^{CT}$ were obtained after one day of vapour diffusion against 10% PEG 6000, 0.1 M HEPES (pH 7.0).

Before data collection, crystals were soaked in cryoprotectant solution containing 20% glycerol and flash-frozen in liquid nitrogen. The X-ray diffraction data were collected at the ESRF beamline ID29 (Grenoble, France) and were indexed and scaled using XDS [28] to a resolution of 3.5 Å and 3.1 Å for the oxidized and reduced forms, respectively.

Structure solution and refinement. The structures of AIF-$G307E^{OX}$ and AIF-$G307E^{CT}$ were solved by the molecular replacement method using the program MOLREP [29] and the corresponding structures of wild type murine AIF (PDB-ID: 3GD3 and 3GD4, respectively) [14] as search models. For both crystals, the two molecules in the crystal asymmetric unit were subjected to rigid-body refinement, and subsequently to constrained refinement

Table 1
X-ray data-collection and refinement statistics.

	AIF-$G307E^{OX}$	AIF-$G307E^{CT}$
Data collection		
Beam line &	ESRF ID29	ESRF ID29
wavelength (Å)	0.97895	0.97895
Space group	$P2_12_12_1$	$P2_12_12_1$
Unit-cell parameters (Å)	a = 96.0; b = 110.1; c = 119.6	a = 73.4; b = 116.8; c = 166.5
Molecules in a.u.	2	2
Resolution (Å)	48.7–3.5	43.5–3.1
Unique reflections	16,448 (1197)[A]	26,576 (1946)[B]
Completeness (%)	99.4 (99.8)	99.5 (99.8)
Redundancy	5.4 (5.7)	4.5 (4.7)
Rmeas[a] (%)	39.3 (129.5)	18.8 (127.8)
CC(1/2) (%)	97.3 (73.5)	99.2 (33.1)
Average I/σ (I)	5.3 (1.8)	6.6 (1.3)
Final model		
R factor[b]/Rfree[c] (%)	21.0/27.8	19.9/25.9
r.m.s. bonds (Å)	0.009	0.010
r.m.s. angles (°)	1.31	1.52
Average protein B fac. ($Å^2$)	73.0	95.0
Residues in most favored regions (%)	94.5%	97.0%
Residues in additionally allowed regions (%)	5.4%	3.0%
PDB-ID	5MIU	5MIV

Values in parentheses are for the highest resolution shell: [A](3.59–3.50), [B](3.18–3.10).
[a] $R_{meas} = (\Sigma\ (n/(n-1)\ \Sigma\ |I - (I)|)/\Sigma\ I$ x 100, where I is intensity of a reflection and (I) is its average intensity.
[b] $R_{factor} = \Sigma\ |F_o - F_c|/\Sigma\ |F_o|$ x 100.
[c] R_{free} is calculated on 5% randomly selected reflections, for cross-validation.

Please cite this article in press as: L. Sorrentino, et al., Structural bases of the altered catalytic properties of a pathogenic variant of apoptosis inducing factor, Biochemical and Biophysical Research Communications (2017), http://dx.doi.org/10.1016/j.bbrc.2017.06.156