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A Redox Reaction in Axon Guidance: Structure and Enzymatic Activity of MICAL

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During development, neurons are guided to their final targets by external cues. MICAL, a large multidomain cytosolic protein, is a downstream signaling molecule required for repulsive axon guidance. We have determined the structure of the N-terminal FAD-binding domain of MICAL to 2.0 Å resolution. This structure shows that $MICAL_{fd}$ is structurally similar to aromatic hydroxylases and amine oxidases. We obtained biochemical data that show that $MICAL_{fd}$ is a flavoenzyme that, in the presence of NADPH, reduces molecular oxygen to H_2O_2. We propose that the H_2O_2 produced by this reaction may be one of the signaling molecules involved in axon guidance by MICAL.

Neurons are required to make path-finding decisions throughout their development and are guided to their final targets by a variety of environmental cues. Semaphorins are a family of guidance molecules that act as repellents in a variety of axon development processes. Repulsive guidance by Semaphorins is mediated through their interaction with Plexins, a family of transmembrane receptors. Biochemical and genetic analysis indicate that a large multi-domain cytosolic protein, MICAL (Molecule Interacting with Cas-L), is required for the repulsive axon guidance mediated by the interaction of Semaphorins and Plexins. MICAL proteins contain a large amino-terminal FAD-binding domain ($MICAL_{fd}$), followed by a series of protein-protein binding domains. $MICAL_{fd}$ is of great interest since it offers a novel link between redox reactions and an axon guidance response. Using x-ray crystallography, we determined the structure of murine MICAL1 FAD-binding domain, $MICAL_{fd}$ (**Figure 1**).

$MICAL_{fd}$ is a mixed α/β globular protein that contains a Rossmann β-α-β fold, two conserved FAD-binding motifs, and a third conserved sequence motif. The first conserved GXGXXG dinucleotide binding motif resides within the Rossman fold. The second conserved GD motif has been observed in flavoprotein hydroxylases and forms part of a strand and a helix. A search of known structures reveals that the $MICAL_{fd}$ protein is most similar to aromatic hydroxylases, especially the *p*-hydroxybenzoate hydroxylase (pHBH) from *P. fluorescens* (rms 1.79 Å for 199 out of 484 aligned α-Carbons).

The strong structural similarity of $MICAL_{fd}$ to PHBH suggests that the two proteins might have similar enzymatic activities. Since purified $MICAL_{fd}$ contains oxidized FAD, reduction of the cofactor was tested using either NADH (nicotinamide adenine dinucleotide) or NADPH (nicotinamide adenine dinucleotide phosphate). Although no net reduction of the FAD was detected, a steady, time-dependent oxidation of reduced nicotinamide dinucleotide was observed (**Figure 2**). This observation suggested that enzyme-bound $FADH_2$ was formed but was then reoxidized by oxygen. The resulting production of H_2O_2 was confirmed by monitoring its formation with horseradish peroxidase in a coupled spectrophotometric assay **(Figure 2)**. The rate of the reaction is

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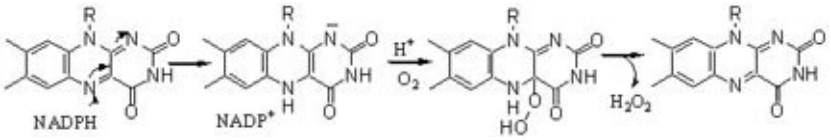
over 10 times faster with 200 μM NADPH than with 200 μM NADH, suggesting that MICAL is probably an NADPH-dependent enzyme.
The observation of this enzymatic activity can be explained by one of three cases. First, H_2O_2 is the physiological product of the enzyme and is a component of the avoidance signal. Second, as with other FAD hydroxylases, in the absence of substrate the hydroperoxide form of the enzyme (the $MICAL_{fd}$-FADH-O_2H intermediate) decomposes, producing hydrogen peroxide and oxidized enzyme-bound FAD (**Scheme 1**). Third, the enzyme may actually be an amine oxidase in which the $FADH_2$ is reoxidized by molecular oxygen, and the reduction by NADPH is a fortuitous, non-specific reaction. Although this last case is unlikely, discrimination among these possibilities requires further experimentation.

The synthesis of a specific metabolite and the production of reactive oxygen species have been previously proposed as possible mechanisms of MICAL signaling. We have shown here that the FAD-binding domain of MICAL can generate at least the second kind of signals: MICAL reduces molecular oxygen using NADPH to produce H_2O_2.



Scheme 1

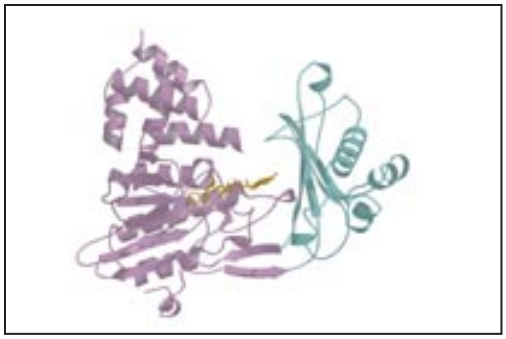


Figure 1. *Ribbon representation of the tertiary structure of $MICAL_{fd}$. $MICAL_{fd}$ is a mixed α/β globular protein composed of two sub-domains of different sizes linked by two β-strands. Subdomain-1 is colored in magenta and subdomain-2 is colored in light blue. The observed FAD molecule is colored in yellow. The large sub-domain (subdomain-1; residues 1 to 226 and 373 to 484) contains the two known FAD sequence motifs (residues 84 to 114 and 386 to 416) and a third conserved motif typically found in hydroxylases (residues 212 to 225). The first motif is part of a Rossmann β-α-β fold (β1-α5-β2 in $MICAL_{fd}$). This is a sequence commonly found in FAD and NAD(P)H-dependent oxidoreductases. The second motif, which contains a conserved GD sequence in hydroxylases, forms part of a strand and a helix. In $MICAL_{fd}$ this second conserved sequence makes contacts with the ribose moiety of FAD.*

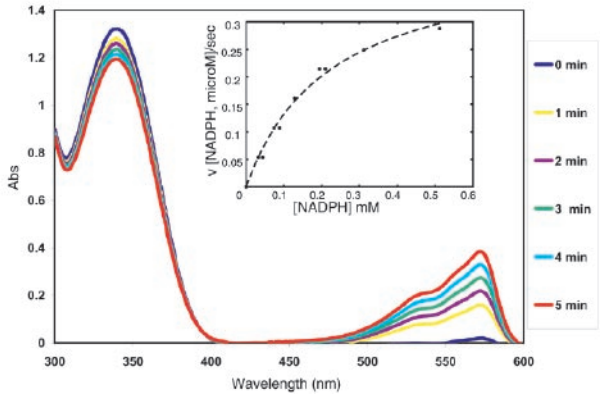


Figure 2. *Kinetics of NADPH oxidation and H_2O_2 production. The absorbance peak at 340 nm is characteristic of reduced NAPDH and the peak at 560 nm the concentration of H_2O_2. The experiment ran for five minutes after the addition of the enzyme. The inset shows the initial rates as a function of NADPH concentration.*