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Site directed spin labeling studies of *Escherichia coli* dihydroorotate dehydrogenase N-terminal extension

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ABSTRACT

Dihydroorotate dehydrogenases (DHODHs) are enzymes that catalyze the fourth step of the *de novo* synthesis of pyrimidine nucleotides. In this reaction, DHODH converts dihydroorotate to orotate, using a flavine mononucleotide as a cofactor. Since the synthesis of nucleotides has different pathways in mammals as compared to parasites, DHODH has gained much attention as a promising target for drug design. *Escherichia coli* DHODH (EcDHODH) is a family 2 DHODH that interacts with cell membranes in order to promote catalysis. The membrane association is supposedly made via an extension found in the enzyme's N-terminal. In the present work, we used site directed spin labeling (SDSL) to specifically place a magnetic probe at positions 2, 5, 19, and 21 within the N-terminal and thus monitor, by using Electron Spin Resonance (ESR), dynamics and structural changes in this region in the presence of a membrane model system. Overall, our ESR spectra show that the N-terminal indeed binds to membranes and that it experiences a somewhat high flexibility that could be related to the role of this region as a molecular lid controlling the entrance of the enzyme's active site and thus allowing the enzyme to give access to quinones that are dispersed in the membrane and that are necessary for the catalysis.

1. Introduction

Dihydroorotate dehydrogenase (DHODH) is a flavin-containing enzyme that catalyzes the oxidation of L-dihydroorotate to orotate, the fourth and only redox step in the *de novo* pyrimidine biosynthesis pathway [1]. DHODH has been considered an important target for the design of antiproliferative and antiparasitic agents once inhibition of DHODH leads to reduced levels of pyrimidine precursors which are important for performing a broad range of cellular functions such as cell growth, metabolism and differentiation [2–4].

For DHODHs, the conversion of dihydroorotate to orotate has been described to follow a sequential ping–pong mechanism [5,6]. In the first half-reaction comprising the reduction of dihydroorotate to orotate, electrons are transferred to the flavine mononucleotide moiety (FMN) which becomes oxidized to dihydroflavin mononucleotide ($FMNH_2$). After dissociation of orotate from the enzyme, $FMNH_2$ is regenerated by an appropriate electron receptor.

Based on amino acid sequence similarity, cell location and substrate specificity, DHODHs from different organisms can be divided in two families [1]. Family 1 members are cytosolic enzymes of Gram-positive bacteria, *Archea* and some unicellular eukaryotes, and is further divided into 1A and 1B depending upon their electron receptor and oligomeric state. Family 1A members are dimeric enzymes and utilize fumarate as the electron receptor. Family 1B enzymes form heterotetramers and utilize NAD^+ via a distinct protein subunit that contains a 2Fe–2S cluster and a FAD cofactor [7]. Enzymes of family 2 exist as monomers and are membrane-bound enzymes that utilize quinones as the physiological oxidant during the second half-reaction. The DHODH in family 2 include the enzyme from *Escherichia coli* (EcDHODH), which is attached to the cell membrane [8,9], as well as the enzymes from higher eukaryotes, e.g., *Homo sapiens*, *Plasmodium falcipurum*, which are localized in the inner membrane of mitochondria [10].

DHODHs fold into an α/β barrel with the central barrel of eight parallel β strands surrounded by eight α helices. Two antiparallel β strands are found at the bottom of the barrel, whereas additional secondary structural elements and loops form a protuberant subdomain present at the top of the barrel [11,12]. The prosthetic FMN group is located between the top of the barrel and the subdomain formed by these insertions. In addition to this main barrel, DHODHs of family 2 contain an extra N-terminal domain situated

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next to the barrel and which folds into two α-helices and one 3_{10} helix [13–15].

Kinetic and structural studies revealed distinct mechanisms for substrate binding when comparing family 1 and 2 DHODHs [16]. Although for all DHODHs the pyrimidine-binding site is found on the si face of the flavin, where the first reductive half-reaction takes place, the oxidative half-reactions for each family are different. In family 1, the dihydroorotate/orotate binding site is also described to be occupied by the oxidant agent [6]. For family 2 members, the N-terminal extension has been proposed to harbor the quinone-binding site, leading the electron acceptor to the FMN cofactor for the redox reaction [13]. This hydrophobic tunnel-like pocket formed by the N-terminal domain has also been found to be the target for drug development [13–17].

To date there is limited information on the mechanism of membrane binding and quinone diffusion in family 2 DHODHs. In our previous work, using *E. coli* DHODH (EcDHODH) as the model system, we have showed that the interaction between family 2 DHODH and model membranes causes a defect-like structure in the membrane hydrophobic region, which is probably the mechanism used by the protein to capture quinones used as electron acceptors during catalysis [18]. In the present work, we have moved forward and investigated the structural changes in the DHODH N-terminal domain in the presence of a membrane model system. To achieve this goal we produced mutants of EcDHODH bearing spin probes in the N-terminal extension. This was done by means of site directed mutagenesis in which a native residue was changed to a cysteine that was in turn specifically labeled with a nitroxide-like probe. This is the basic idea governing the so-called site directed spin labeling technique that, along with ESR experiments, allow one to characterize dynamics and conformational changes occurring during protein function [19–22]. Our results give clear experimental evidences that the N-terminal extension is indeed responsible for the association with the membrane, a paramount step for the enzyme catalysis, and also shed light on the conformational dynamics experienced by that region in presence of the membrane model.

2. Materials and methods

2.1. Materials

1-Dipalmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (DOPC) was obtained from Avanti Polar Lipids, Inc. (Birmingham, AL). The sulfhydryl reactive spin-label (1-oxyl-2,2,5,5-tetramethyl-3-pyrroline-3-methyl) methanethiosulfonate (MTSL) was purchased from Toronto Research Chemicals, Inc. (North Fork, ON).

2.2. Construction, expression, purification and spin labeling of EcDHODH mutants

The template EcDHODH gene used for mutagenesis was that coding for the cysteine-free "pseudo-wild-type" protein containing the substitutions C48A, C70A, C158A, and C260A (Mutagenx, NJ). Single cysteine residues were introduced at positions Y2, F5, H19, and F21 of EcDHODH using three-step PCR method. Mutations were confirmed by nucleotide sequencing [23].

EcDHODH mutants were expressed in *E. coli* strain SO6740 [24]. Protein expression was induced by addition of 750 μM isopropyl β-D-thiogalactopyranoside (IPTG). The cells were grown overnight in Luria-Broth medium at 37 °C with vigorous shaking. The cells were collected by centrifugation at 4000*g* for 30 min. EcDHODH mutants were purified as described for the wild-type enzyme [18]. Each EcDHODH mutant was tested to determine whether they were stable and active. The activity test of the EcDHODH mutants was performed as described by Bjornberg et al. [24]. The mutants were then spin labeled with a 10-fold molar excess of the MTSL spin-label after anion exchange chromatography. Spin labeling proceeded overnight at 4 °C under gentle shaking. The unbound label molecules were removed by using dialysis through centrifugal filter devices.

2.3. ESR spectroscopy

Aliquots of a chloroform stock solution of the phospholipid DOPC were dried inside a glass tube by using a nitrogen flow. The dried film was resuspended with the solution containing spin-labeled protein mutants. The final solution for each mutant (50–200 μM) was drawn into a quartz flat cell for ESR experiments. ESR spectra were acquired using a Varian E109 X-band (9.4 GHz) spectrometer. All experiments were carried out at room temperature and several scans were performed to achieve a good signal-to-noise ratio. Experimental parameters were set to avoid saturation and distortions of the final spectrum and included: magnetic field range of 100 G, microwave power of 20 mW, modulation amplitude of 1.0 G.

2.4. NLSL simulations

Non-linear least-squares simulations were performed using the NLLS software, which is based on Freed's developments of the Stochastic Liouville Equation (SLE) [25,26] and implemented in the LabView graphical interface made available by Dr. Christian Altenbach (UCLA) at https://sites.google.com/site/altenbach/labview-programs/epr-programs/multicomponent. The routine solves the SLE for a nitroxide radical undergoing a diffusion motion in a complex fluid and has been successfully applied to a variety of cases [27–29], allowing the calculation of a theoretical ESR spectrum. In terms of parameters, the outputs are: rotational diffusion rates around axes defined in a molecular reference frame and expressed as the components of an R-tensor (in this paper, we used $\bar{R}$ and N that are the components of the tensor in a spherical representation with $\bar{R}$ being the geometric mean $\bar{R} = \bar{R}_x\bar{R}_y\bar{R}_z$ and N the axial rotational anisotropy $N \equiv R_z/\sqrt{R_xR_y}$); order parameter S, calculated from the coefficients of a restoring potential (c20, c22) that governs the diffusion of the probe in a medium with microscopic ordering; line broadenings that can be Gaussian (Δ_G) or Lorentzian (W) and take into account contributions due to inhomogeneous broadenings. All parameters and their definitions are well described in the seminal paper by Budil et al. [26]. The magnetic tensors that describe the Zeeman (g-tensor) and hyperfine (A-tensor) interactions are input parameters taken from the literature in cases where similar structural environments to those found in this work were described. These magnetic parameters are allowed to vary only at the end of the simulation routines as a way of getting the finest adjustment of the experimental spectra. In the cases where a multi-component spectrum is being simulated the program also gives the percentage population for each component.

3. Results and discussions

EcDHODH contains four native cysteine residues that needed to be replaced in order to achieve the specificity required for site directed spin labeling experiments. Hence the first step in our study was to produce a pseudo-native enzyme where each cysteine was mutated to an alanine residue. Previous studies on class2 DHODHs have shown that protein structure and stability are not dependent of N-terminal extension. Truncated versions of malarial DHODH have already been used in order to kinetically characterize the enzyme and search for new inhibitors [30,31]. In fact, the N-terminal