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MdaB from *Escherichia coli*: cloning, purification, crystallization and preliminary X-ray analysis

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The gene *mdaB* from *Escherichia coli* encodes an enzyme with activity similar to that of mammalian DT-diaphorase. It has been reported that the protein is able to confer resistance to the antibiotics DMP 840, adriamycin and etoposide. The gene was cloned and overexpressed in *E. coli*, allowing purification of the protein to homogeneity. The protein co-purified with an unidentified flavin. Suitable crystals for X-ray diffraction experiments were obtained by hanging-drop vapour diffusion. Their space group was triclinic *P*1, with unit-cell parameters $a = 48.664$, $b = 52.099$, $c = 86.584$ Å, $\alpha = 87.106$, $\beta = 86.889$, $\gamma = 63.526^\circ$. X-ray diffraction data were collected to 2.5 Å.

1. Introduction

Quinones are biologically active molecules that function as lipid electron carriers, transporting hydrogen and electrons between the protein complexes of the electron-transport chain. Ubiquinone, found in the mitochondria of eukaryotic cells, and menaquinone, a derivative of vitamin K predominantly employed during anaerobic respiration, are both synthesized by *Escherichia coli*. While these compounds are essential for normal electron transport, it has been demonstrated that quinonoid compounds are capable of diverting the electron flow from the respiratory chain of *E. coli*, causing increased intracellular production of superoxide radicals and hydrogen peroxide (Hassan & Fridovich, 1979). Benzoquinones and naptho-quinones, such as menaquinone, have much higher redox potentials than many other quinones. Their high electrophilicity and thiol reactivity enable rapid reduction to semiquinone intermediates (O'Brien, 1991). The toxicity of these and similar compounds is such that they are frequently employed as chemotherapeutic agents and many quinone derivatives from Gram-positive bacteria manifest substantial biological activity in terms of anticancer, antimicrobial and cytotoxic effects resulting from the oxidation of cellular macro-molecules (Bolton *et al.*, 2000).

The metabolism and toxicity of menadione (2-methyl-1,4-napthoquinone) have been extensively studied in mammalian cells (Brunmark *et al.*, 1987; Iyanagi & Yamazaki, 1970). DT-diaphorase, an NAD(P)H:oxidoreductase, is believed to reduce a variety of quinone substrates by means of a two-electron reduction mechanism. It has been demonstrated that the activity of this enzyme protects rat hepatocytes from menadione toxicity by competing with the potentially toxic one-electron reduction pathway employed by the electron-transport chain and avoiding the generation of a semiquinone intermediate (Lind *et al.*, 1982, 1990; Thor *et al.*, 1982). The enzyme 'modulator of drug activity B' (MdaB) was first identified on the basis of the protection it provided from the toxic effects of DMP 840, adriamycin and etoposide when overexpressed in *E. coli* (Chatterjee & Sternberg, 1995). It was later confirmed through protein sequencing that this protein corresponded to a DT-diaphorase-like enzyme previously purified from *E. coli* that demonstrated reductase activity toward menadione (Hayashi *et al.*, 1996). MdaB expression was reported to be up-regulated more than 20-fold in the cytoplasmic fraction of *E. coli* in response to 0.2–0.3 m*M* menadione (Hayashi *et al.*, 1990). While early work suggested FMN- and NADH-dependence, later studies found the protein to be

FAD- and NADPH-dependent (Hayashi *et al.*, 1996). Confusion remains, however, as to the identity of the flavin employed by MdaB. It has been suggested that induction of this enzyme may be an adaptive response to minimize the toxicity of menadione. Recent work in our laboratory has pointed to a shared role for MdaB in enzymatic quinone redox cycling along with the protein YgiN and we now refer toYgiN as quinol monooxygenase (QuMo). It is possible that such a redox cycle allows the cell to maintain a stable pool of quinones for electron transport while attenuating the potential toxicity of both quinone and quinol species. Additional structural and biochemical work will be required in order to further investigate such a role for both proteins.

2. Materials and methods

2.1. Cloning procedure, expression and purification

The *mdaB* ORF was amplified by polymerase chain reaction using the *E. coli* O157:H7 EDL933 genomic DNA as a template and the forward and reverse primers 5′-AAAAAAGGATCCAGCAACATCCTGATTATCAACGGC and 5′-AAAAAAGAATTCTTAACCAAAAATTTCCACAAGATGCTT, respectively. The amplified DNA was inserted into the pFO4 plasmid (Novogen, Madison, WI, USA) to yield the pFO4-His_6-*mdaB* construct. The plasmid was sequenced to ensure no mutations had been introduced during the amplification reaction.

E. coli BL21 (DE3) transformants containing the pFO4-His_6-*mdaB* construct were grown in 5 ml aliquots and then subcultured into 1 l of fresh Terrific Broth (Bioshop Canada Inc.) supplemented with ampicillin (200 µg ml^{-1}) and grown to an A_{600} of 0.9 at 310 K. Protein expression was then induced for 6 h with 0.1 m*M* isopropyl-β-thiogalactoside and cells were harvested by centrifugation (20 min, 3297*g*). The cell pellet was then resuspended in 50 m*M* sodium phosphate pH 8.0, 0.1% Triton X-100 and 0.3 *M* NaCl and cells were disrupted by sonication. Following centrifugation (40 min, 20 070*g*) at 277 K, the supernatant was incubated for 90 min at 277 K with 5 ml Ni–NTA resin (Qiagen) that had been pre-equilibrated with 50 m*M* sodium phosphate pH 8.0 and 0.3 *M* NaCl. The supernatant-resin slurry was then loaded into a 25 ml column and the flowthrough collected. A washing step with ten column volumes of buffer containing 50 m*M* sodium phosphate pH 8.0 and 0.3 *M* NaCl was performed. The protein was eluted from the column with 50 m*M* sodium phosphate pH 8.0, 0.3 *M* NaCl and 100 m*M* imidazole.

At this stage, wild-type MdaB protein was pure as checked by SDS–polyacrylamide gel followed by Coomassie Brilliant Blue R-250 staining. The yield was estimated to be 200 mg per litre of culture. The observed monomeric weight was approximately 22 000 Da as observed by SDS–PAGE. The MdaB protein appears to form homodimers in solution as an apparent molecular weight of 44 000 Da was obtained from dynamic light-scattering measurements using a DynaPro MSX/TC from Protein Solutions (data not shown). Surprisingly, the purified protein displayed an intense yellow colour,

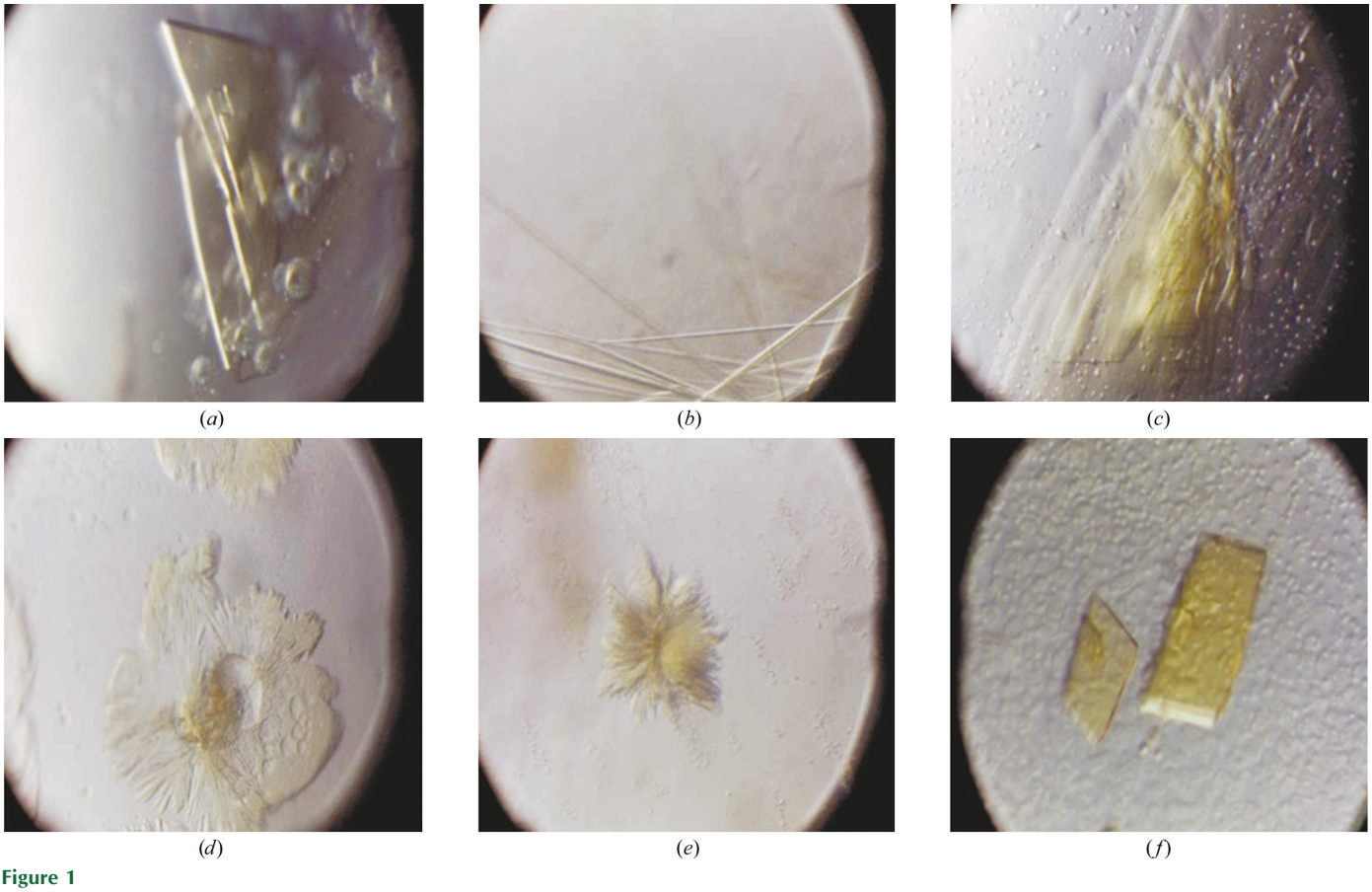


Figure 1
Crystals obtained under various conditions from commercially available screens. The crystallization conditions were (*a*) 0.2 *M* magnesium acetate tetrahydrate, 0.1 *M* sodium cacodylate pH 6.5, 20% polyethylene glycol 8000, (*b*) 0.1 *M* sodium acetate trihydrate pH 4.6, 8% polyethylene glycol 4000, (*c*) 0.1 *M* HEPES sodium salt pH 7.5, 2% polyethylene glycol 400, 2.0 *M* ammonium sulfate, (*d*) 0.2 *M* ammonium sulfate, 0.1 *M* MES pH 6.5, 30% polyethylene glycol monomethylether 5000, (*e*) 1.6 *M* trisodium citrate dihydrate pH 6.5 and (*f*) the refined condition 0.15 *M* magnesium acetate tetrahydrate, 0.1 *M* sodium cacodylate pH 6.5, 20% polyethylene glycol 8000.

the absorbance spectrum of which was consistent with the co-purification of the flavin cofactor (data not shown).

2.2. Crystallization

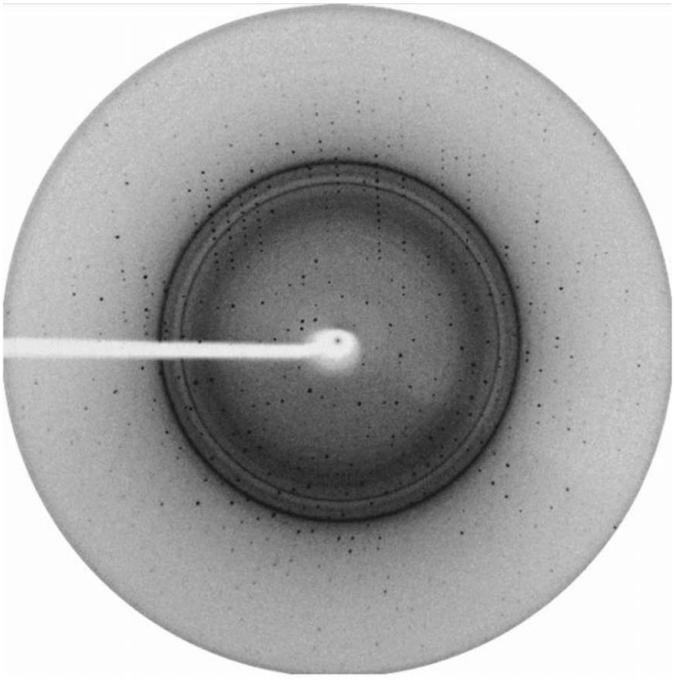
The purified enzyme was concentrated using a Centricon (Amicon/Millipore) with a 10 kDa molecular-weight cutoff at 997*g* and 277 K. The imidazole was removed by buffer exchange during concentration. Crystallization experiments were performed using the hanging-drop vapour-diffusion method at room temperature. Drops were prepared by mixing 2 µl protein solution with an equal volume of reservoir solution and were suspended over 500 µl reservoir solution. Initial crystallization screens were carried out at a protein concentration of 10 mg ml^{-1} using Crystal Screens I and II from Hampton Research. The initial crystallization conditions were further refined.

Table 1
Diffraction data for native MdaB crystals.

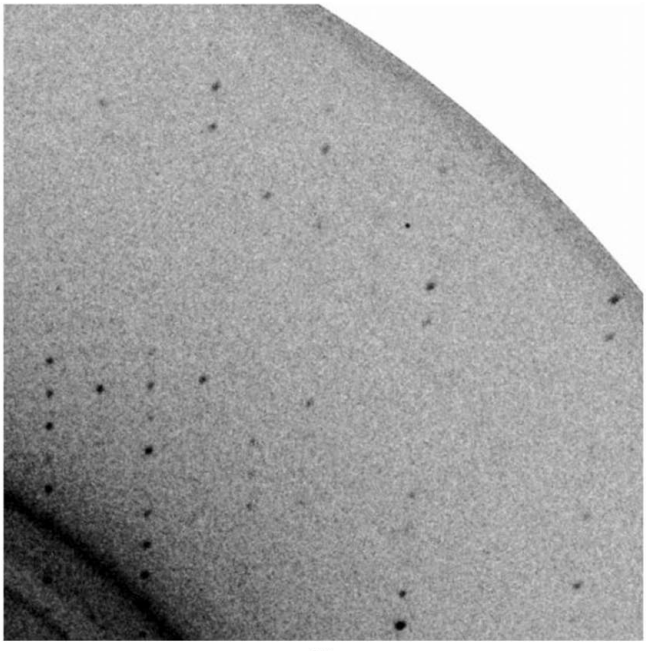
Data in parentheses are for the high-resolution shell (2.59–2.5 Å).

Temperature (K)	100
Resolution (Å)	50.0–2.50
No. of observations/No. of unique observations	35393/23155
Completeness	85.7 (78.2)
$\langle I/\sigma(I)\rangle$	16.79 (5.62)
$R_{\rm sym}$†	0.044

† $R_{\rm sym} = \sum |I(k) - \langle I\rangle| / \sum I(k)$, where $I(k)$ and $\langle I\rangle$ represent the diffraction-intensity values of the individual measurements and the corresponding mean values. The summation is over all measurements.



(*a*)



(*b*)

Figure 2
(*a*) Diffraction of native MdaB crystals grown in 0.15 *M* magnesium acetate, 0.1 *M* sodium cacodylate pH 6.5, 20% PEG 8000. Diffraction data were indexed and reduced to 2.5 Å with good statistics. However, diffraction is apparent even at 2.3 Å at the edge of the detector (*b*).

2.3. Data collection and diffraction measurements

MdaB crystals suitable for X-ray data collection were mounted in light mineral oil as a cryoprotectant and brought to cryogenic temperature in an Oxford Cryosystems Cryostream. Native crystal data were collected using the in-house facility composed of a Rigaku copper rotating-anode X-ray generator and a MAR Research imaging plate. The crystal-to-detector distance was set at 175 mm with 0.25° oscillation and 75 s exposure per image. All data sets were indexed and integrated with *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997).

3. Results and discussion

Recombinant MdaB protein was purified to homogeneity using a one-step procedure. The nickel-affinity column produced protein of very high purity that was sufficient for crystallization trials. This MdaB preparation displayed typical menadione reductase activity without cleavage of the N-terminal histidine purifiaction tag. Initial crystallization conditions were obtained from conditions 18 and 37 of Crystal Screen I and conditions 26 and 28 of Crystal Screen II (Fig. 1). Subsequent optimization of condition 18 from Crystal Screen I yielded then clustered plate-like crystals that were suitable for diffraction experiments. The final crystallization condition contained 0.15 *M* magnesium acetate, 0.1 *M* sodium cacodylate pH 6.5, 20% PEG 8000 using a starting protein concentration of 10 mg ml^{-1} in 50 m*M* NaH_2PO_4 pH 8.0 and 300 m*M* NaCl. MdaB crystals grown from this condition have typical dimensions of 0.05 × 0.3 × 0.4 mm.

The diffraction pattern obtained using the in-house facility was consistent with triclinic *P*1 symmetry and produced unit-cell parameters a = 48.664, b = 52.099, c = 86.584 Å, α = 87.106, β = 86.889, γ = 63.526° (Fig. 2). A total of 35 393 reflections were collected (with an average multiplicity of 1.5) in the resolution range 50–2.5 Å (Table 1). Crystal mosaicity was calculated to be 0.85° for in-house data collection for several crystals. A reasonable Matthews coefficient of 2.2 Å^3 Da^{-1} (Matthews, 1968) was obtained for four monomers in the asymmetric unit. The resulting calculated solvent content is 44%. The self-rotation function was calculated using *POLARRFN* from the *CCP*4 package (Collaborative Computational Project, Number 4, 1994). An unambiguous peak was observed at (ψ = 0,

φ = 29.0, κ = 180°) with 86% of the origin-peak intensity, consistent with the presence of a non-crystallographic twofold axis relating two dimers of MdaB in the asymmetric unit.

MdaB displays 25% sequence identity to other NAD(P)H: oxidoreductases whose crystal structures have already been determined. Consequently, a molecular-replacement search using monomeric and dimeric search models has been attempted using a variety of molecular-replacement programs. However, no solutions have been found. We have therefore generated selenomethionine-labelled protein and are currently optimizing the crystallization conditions for subsequent MAD data collection and structure solution.

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