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Crystallization and preliminary crystallographic studies of chloroplast NADP-dependent malate dehydrogenase from *Flaveria bidentis*

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Abstract

Crystals of chloroplast NADP-dependent malate dehydrogenase have been grown both with and without the cofactor NADP present. The enzyme has a molecular weight of 43 kDa per subunit and exists as a dimer in solution. The crystals diffract to 2.8 Å and belong to the space group $P3_221$ with cell dimensions a = 148.1, c = 65.5 Å.

1. Introduction

NADP–malate dehydrogenase (E.C. 1.1.1.82) is a chloroplast enzyme that undergoes rapid reversible light-dependent activation (Edwards *et al.*, 1985). The enzyme is part of the C_4 pathway of photosynthesis in monocot plants such as maize, sugar cane and sorghum and the dicot plants such as *Flaveria bidentis*. The light activation is achieved by photosynthetic electron transport-dependent reduction of enzyme disulfide bonds. The activation can be mimicked *in vitro* by treatment of the enzyme in extracts with thiol reducing agents such as dithiothreitol or the reduced form of the protein thioredoxin. Similarly the deactivation that occurs in darkened leaves can be mimicked by thiol oxidants. This thiol-dependent regulation of activity is also a property of the purified enzyme. The purified oxidized enzymes from both maize and *F. bidentis* are essentially completely inactive but can be activated many 1000-fold by thiol reducing agents (Ashton & Hatch, 1983*a*; Trevanion & Ashton, 1998). Covalent regulation of enzyme activity by reversible reduction and re-oxidation of regulatory disulfide bonds accounts for the light-dependent activation of at least six chloroplast enzymes (Buchanan, 1991). This regulatory mechanism seems to function as a chloroplast equivalent of covalent regulation by protein phosphorylation that occurs in all organisms. Chloroplast NADP–malate dehydrogenases are homologous to NAD–malate dehydrogenases that occur in the cytoplasm and other organelles of bacteria, plants and animals but, in addition, contain N- and C-terminal polypeptide extensions that are not found in the NAD-dependent enzymes. These extensions contain conserved cysteines that are believed to be the regulatory disulfides that mediate the light- and redox-dependent activation of NADP–malate dehydrogenase (Miginiac-Maslow *et al.*, 1997). The cysteines in the terminal extensions are not essential for catalytic activity since they can be all mutated to Ala or Ser with little or no loss of catalytic activity. However, such mutant enzymes are now permanently active and resistant to thiol oxidants (Issakidis *et al.*, 1994). The molecular basis for the dramatic change in activity between oxidized and reduced NADP–malate dehydrogenase is not known although the change is accompanied by a 100-fold change in the relative binding affinities for $NADP^+$ and NADPH. The coenzyme $NADP^+$ also profoundly alters the redox potential of one of the regulatory dithiol/disulfide groups (Ashton & Hatch, 1983*b*) which may have significance for the light-dependent regulation of the enzyme *in vivo* (Trevanion *et al.*, 1997; Furbank *et al.*, 1997).

We have recently purified and cloned the NADP–malate dehydrogenase from the C_4 plant *F. bidentis* (Trevanion & Ashton, 1998). Here we report the crystallization and preliminary X-ray analysis of the protein in its oxidized form.

2. Purification of NADP–malate dehydrogenase

NADP–malate dehydrogenase was purified from darkened leaves of *F. bidentis* essentially as described previously for the enzyme from maize (Ashton & Hatch, 1983*a*; Ashton *et al.*, 1990). Briefly purification involves ammonium sulfate fractionation, polyethylene glycol fractionation and pH precipitation followed by dye-ligand affinity chromatography on reactive red 4-Sephacryl S-300 and gel filtration through Sephacryl S-300 (Trevanion & Ashton, 1998). The enzyme was purified as the oxidized form in the absence of thiol-reducing agents and could be activated more than 10 000-fold by incubation with dithiothreitol and thioredoxin to a specific activity of more than 400 μmol min $^{-1}$ (mg protein) $^{-1}$. N-terminal amino-acid sequencing showed that the purified enzyme consisted of two species. The sequence of the longer species began at residue 69 of the primary translation product (SwissProt entry: MDHC_FLABI) while the second species started at residue 79. The longer form is the full-length mature chloroplast enzyme after removal of the transit peptide.

3. Crystallization

The Hampton Crystal screen II which is based on the sparse-matrix sampling of Jancarik & Kim (1991) was used to test 48 crystallization conditions. The screen was carried out at 277 K using 2 μl of protein solution at a concentration of 10.6 mg ml $^{-1}$ or protein solution and NADP (1:4 molar ratio), added to 2 μl of reservoir in a sitting drop set up in Crystal Clear Strips from Hampton Research. From the screens three different conditions produced crystals to a size of approximately 0.25 × 0.22 × 0.18 mm over a period of 6–12 d. All the conditions contained ammonium sulfate, together with PEGMME (polyethylene glycol monomethyl ether) 5 K at pH 6.0, K^+/Na^+ tartrate at pH 5.6 or glycerol at pH 8.5. Screening around these conditions continued at 277 and 291 K in Linbro

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multiwell tissue-culture plates, with 4 µl of protein added to 4 µl of reservoir in a hanging drop suspended over a 500 µl reservoir. Crystals suitable for X-ray diffraction studies were grown at 291 K in 6 d from a reservoir solution consisting of 1.5 *M* ammonium sulfate, 0.1 *M* HEPES pH 7.5 and 12% glycerol. Crystals grown under these conditions can be reproducibly grown to a size of 0.40 × 0.38 × 0.35 mm (Fig. 1).

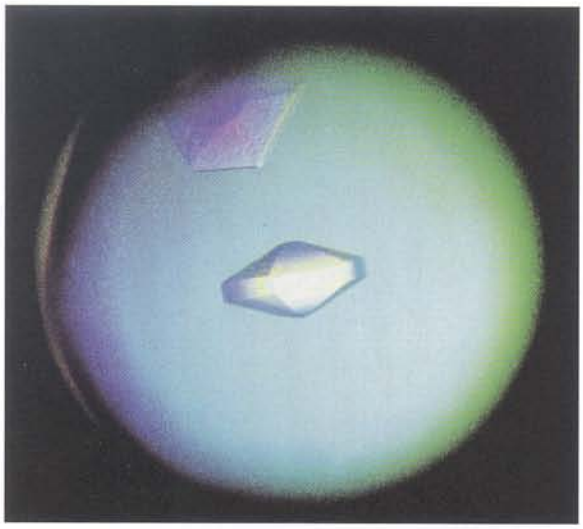

Fig. 1. Photograph of the crystal.

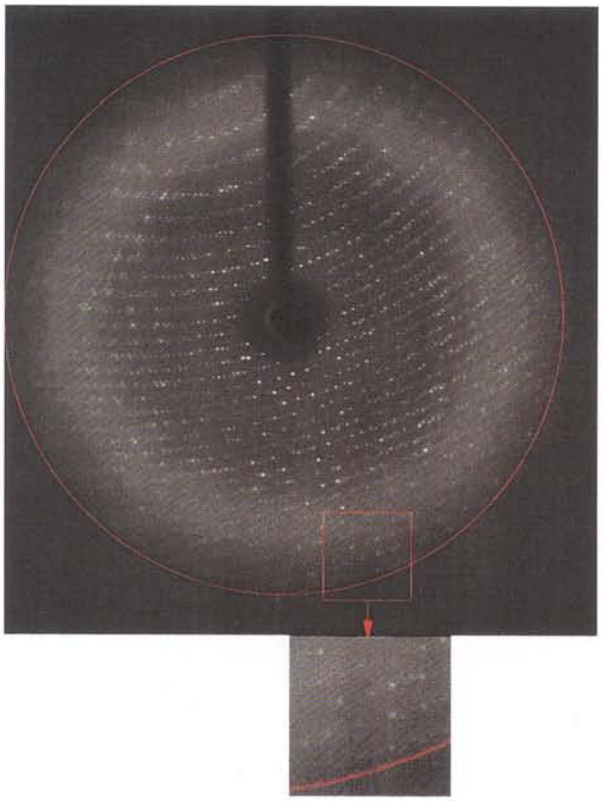

Fig. 2. X-ray diffraction image from a 2° oscillation of the crystal. The red line indicates the 2.8 Å diffraction limit. The insert shows a close up in the region of this limit.

4. Data collection and analysis

X-ray data were collected from two crystals. The first, at room temperature, from a crystal mounted directly from its mother liquor into a standard quartz glass capillary. The second crystal was soaked in a cryobuffer equivalent to the mother liquor but with the glycerol concentration increased to 30%. This second crystal was flash cooled in a nitrogen gas stream at 100 K using the modifications to a Rigaku Raxis-II detector described by Carr *et al.* (1996). The X-ray generator was producing Cu $K\alpha$ radiation at a power of 5 kW (50 kV, 100 mA) in both cases.

The data were processed using the program *HKL* (Minor, 1993; Otwinowski, 1993). The room-temperature data set comprised of 18 788 observations of 7262 unique reflections (66.0% to 3.5 Å) with R_{sym} = 0.083 and $\langle I/\sigma(I)\rangle = 10.4$ in space group $P3_221$ (or related space groups $P321$, $P3_121$). Post refined values of the unit cell were a = 149.9, c = 65.6 Å. The low-temperature data set comprised of 173 660 observations of 20 422 unique reflections (99.7% complete to 2.8 Å) with R_{sym} = 0.077 and $\langle I/\sigma(I)\rangle = 13.2$. Post-refined values of the unit-cell dimensions were a = 148.1, c = 65.5 Å. The diffraction pattern obtained from a 2° oscillation of the second crystal at low temperature is shown in Fig. 2.

The value of the Matthews (Matthews, 1968) coefficent of 2.4 Å^3 Da^{-1} for two subunits per asymmetric unit is consistent with the dimeric nature of the NAD-dependent malate dehydrogenase (Birktoft *et al.*, 1989). However, self-rotation function calculations did not reveal any significant peaks corresponding to a non-crystallographic dimer axis. The self-rotation calculations were performed using a variety of data shells and Patterson cutoff spheres. Calculations were performed using the programs *POLARRFN* and *AMoRe* (Navaza, 1994) as implemented in the *CCP*4 program suite (Collaborative Computational Project, Number 4, 1994). This suggests that the dimer axis may be crystallographic with only one subunit per asymmetric unit. Such an arrangement would lead to a high but not unprecedented value of 4.8 Å^3 Da^{-1} for the Matthews coefficent.

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