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Structural studies of MIP synthase

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The conversion of glucose 6-phosphate to 1-L-*myo*-inositol 1-phosphate (MIP) by 1-L-*myo*-inositol 1-phosphate synthase (MIP synthase) is the first committed and rate-limiting step in the *de novo* biosynthesis of inositol in all eukaryotes. The importance of inositol-containing molecules both as membrane components and as critical second messenger signal-transduction species make the function and regulation of this enzyme important for a host of biologically important cellular functions including proliferation, neurostimulation, secretion and contraction. MIP synthase has been overexpressed in *Escherichia coli* and purified to homogeneity by chromatographic methods. Two crystal forms of MIP synthase were obtained by the hanging-drop vapor-diffusion method. Native data sets for both crystal forms were collected in-house on a Rigaku R-AXIS IIC imaging-plate detector. Crystal form I belongs to space group $C2$, with unit-cell parameters $a = 153.0$, $b = 96.6$, $c = 122.6$ Å, $\beta = 126.4^\circ$, and diffracts to 2.5 Å resolution. Crystal form II belongs to space group $P2_1$, with unit-cell parameters $a = 94.5$, $b = 186.2$, $c = 86.5$ Å, $\beta = 110.5^\circ$, and diffracts to 2.9 Å resolution.

1. Introduction

myo-Inositol is the starting material for the biosynthesis of a host of critically important signaling molecules, including the polyphosphorylated *myo*-inositols and the precursor phosphoinositides (Arganoff & Fisher, 1991). *myo*-Inositol 1-phosphate synthase (MIP synthase, E.C. 5.5.1.4) converts D-glucose 6-phosphate to *myo*-inositol 1-phosphate. This is the first committed and rate-limiting step in the *de novo* biosynthesis of all inositols in eukaryotes (Majumder *et al.*, 1997). This complex transformation occurs *via* a multi-step reaction mechanism involving an oxidation, a reduction and an intramolecular aldol condensation. This mechanism occurs stereospecifically within a single active site with no dissociation of intermediates. The mechanism of the aldol condensation is novel in that it seems to involve neither lysine Schiff-base formation nor metal activation (Loewus & Loewus, 1973; Mauck *et al.*, 1980; Pittner & Hoffmann, 1978; Sherman *et al.*, 1981).

MIP synthase is found in all eukaryotes so far investigated, including protozoa, fungi, algae, plants and mammals. The gene encoding MIP synthase has been cloned from several organisms including yeast (*INO1* gene), amoebas and several plants (Abu-Abied & Holland, 1994; Dean-Johnson & Henry, 1989; Ishitani *et al.*, 1996; Johnson, 1994; Johnson & Burk, 1995; Smart & Fleming, 1996; Wang & Johnson, 1995). In all organisms, MIP synthase codes for a ~60 kDa monomer polypeptide. Very homologous partial sequence data has also been obtained, *via* a BLAST search, from mice and humans (Altschul *et al.*, 1997).

MIP synthase is a member of a unique class of enzymes that use NAD not as a cosubstrate, but as a co-catalyst (Loewus *et al.*, 1984; Loewus & Kelly, 1962). NAD is reduced to NADH and then reoxidized back to NAD in the same catalytic cycle. Though a mechanistic scheme has been proposed, there is no structural data illuminating the details of this enzyme's precise role in this important conversion. There are no mutational data that indicate the location of the active site within the primary sequence, nor has the NAD-binding pocket been identified. Additionally, the three-dimensional fold of the protein is unknown, as the enzyme's amino-acid sequence has no apparent homology to proteins for which the structure is known.

Interestingly, MIP synthase may be a target of therapeutic importance. Currently, lithium is used in the treatment of manic depression (Belmaker *et al.*, 1996; Berridge *et al.*, 1982; Berridge & Irvine, 1989). The reasoning behind this is that lithium acts by inhibiting MIP phosphatase and reducing inositol levels in the brain. Inhibiting MIP synthase may also lower inositol levels in the brain and have a similar therapeutic effect. Other inhibitors of the phosphatase have already been tested in

animal models and are found to have similar effects to those of lithium, adding credence to this mode of action (Koffman *et al.*, 1991; Koffman & Belmaker, 1990).

Here, we report the purification, crystallization and preliminary X-ray diffraction studies of two crystal forms of a key enzyme in the biosynthesis of inositols, MIP synthase.

2. Materials and methods

2.1. Expression, purification and characterization

Yeast MIP synthase has been overexpressed in *E. coli* in the efficient BL21 (DE3) overproducing strain. MIP synthase was expressed following a 3 h induction with 60 mg l^{-1} of IPTG (dioxane-free). Cell pellets were resuspended in buffer *A* (20 m*M* NH_4Cl, 10 m*M* Tris, 10 m*M* β-mercaptoethanol) in a volume proportional to 2 ml buffer *A* per gram of dry cells and stored at 193 K.

After thawing on ice for 30 min, the cells were sonicated in (three) 1 min intervals and centrifuged at 4000 rev min^{-1} for 10 min. The resulting supernatant was purified to homogeneity using four chromatography steps. (i) Phast-Q chromatography (Pharmacia): protein was eluted using a linear salt gradient (high, 300 m*M* NH_4Cl, 10 m*M* Tris, 10 m*M* β-mercaptoethanol; low, 20 m*M* NH_4Cl, 10 m*M* Tris, 10 m*M* β-mercaptoethanol). The fractions containing MIP synthase were pooled and diluted with buffer *A* to lower the salt concentration. (ii) Anion-exchange chromatography: diluted fractions from (i) were applied to a SOURCE-Q column (8 ml total volume, Pharmacia) in buffer *A* (20 m*M* NH_4Cl, 10 m*M* Tris, 10 m*M* β-mercaptoethanol, 10% glycerol). Elution with a linear gradient from buffer *A* to buffer *B* (1 *M* NH_4Cl, 10 m*M* Tris, 10 m*M* β-mercaptoethanol, 10% glycerol) gave peak fractions which were pooled and concentrated to 2 ml for gel filtration. (iii) Gel-filtration chromatography: after concentration, the protein was applied to a gel-filtration column (Superdex 200 16/75, Pharmacia) and eluted in the original buffer *A* (without glycerol). MIP synthase eluted from this column as a 240 kDa tetramer, consistent with previous reports (Donahue & Henry, 1981; Migaud & Frost, 1995). (iv) Blue A affinity chromatography: pooled fractions from (iii) flowed through the affinity resin to remove the residual endogenous glucose 6-phosphate dehydrogenase that co-purifies with MIP synthase. The enzyme was then concentrated to 10 mg ml^{-1} for crystallization experiments.

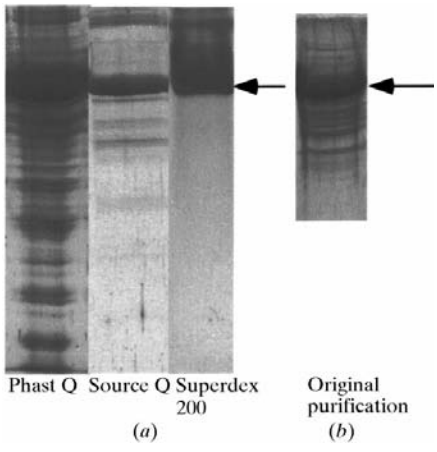

Figure 1
Purification of MIP synthase. (*a*) SDS–PAGE Coomassie-stained gel of MIP synthase after each chromatographic step. (*b*) Purity of MIP synthase using the previous method (Donahue & Henry, 1981; Migaud & Frost, 1995). The arrow corresponds to MIP synthase (64 kDa).

MIP synthase was further characterized by an enzyme-activity assay as previously described (Donahue & Henry, 1981; Migaud & Frost, 1996). This assay involved incubation of MIP synthase with 5 m*M* glucose 6-phosphate and 1 m*M* NAD. Aliquots were removed every 2 min, added to TCA, incubated with aqueous $NaIO_4$ for 1 h at 310 K and quenched with Na_2SO_3. Released inorganic phosphate was determined colorimetrically (Ames, 1966). Defining one unit of activity as 1 μM of MIP for per minute per milligram of enzyme at 310 K, our value of 0.26 μM min^{-1} mg^{-1} compares well with typical values in the literature of 0.22 μM min^{-1} mg^{-1} (Migaud & Frost, 1996). The present purification gives as much as a threefold improvement in purification over previous methods (Donahue & Henry, 1981; Migaud & Frost, 1995) and produces enzyme of superior purity (Fig. 1).

2.2. Crystal growth and analysis

Crystals were grown from hanging drops using the vapor-diffusion technique at room temperature (Wlodawer & Hodgson, 1975). The drops were prepared by mixing 2 µl of protein solution (10 mg ml^{-1} in buffer *A*) with 2 µl of reservoir solution. The drops were equilibrated against 1 ml of reservoir solution. For crystallization of form I, MIP synthase was equilibrated against a reservoir solution containing 2–5%(*v*/*v*) PEG 8000 and 100 m*M* sodium acetate pH 4.5. For crystal form II, MIP synthase was again equilibrated against a reservoir solution containing 5–8%(*v*/*v*) PEG 8000 and 100 m*M* sodium acetate pH 5.0–6.0. Crystals from both forms were observed in 1–2 d.

For data collection, both forms can be transferred to cryoprotectant mother liquors (5% PEG 8000, 100 m*M* sodium acetate at pH 4.5 or pH 5.0, depending on crystal form, and 30% glycerol). Complete data sets were collected at 123 K from a single flash-frozen crystal of each form.

Native data were collected on an R-AXIS IIC imaging-plate system with Cu $K\alpha$ X-rays generated with a Rigaku RU-200 rotating-anode generator operated at 50 kV and 100 mA. Data reduction and scaling were performed using *DENZO* and *SCALEPACK*, respectively (Otwinowski & Minor, 1997).

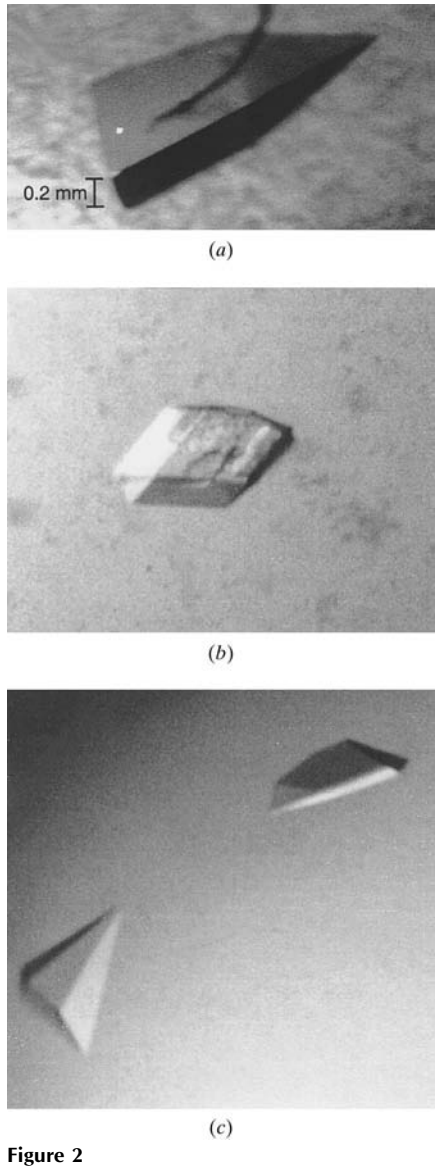

Figure 2
Crystal images. (*a*) MIP synthase crystal form I. Note: crystal nucleated around a fiber. (*b*) MIP synthase crystal form II. (*c*) MIP synthase crystals produced *via* microseeding at a dilution of $10^{-6.5}$.

Table 1
Data-collection statistics.

	Form I	Form II
X-ray source	Rigaku RU-200 50 kV, 100 mA	Rigaku RU-200 50 kV, 100 mA
Space group	*C*2	$P2_1$
Wavelength (Å)	1.54	1.54
Unit-cell parameters		
a (Å)	153.0	94.5
b (Å)	96.6	186.2
c (Å)	122.6	86.5
β (°)	126.4	110.5
Mosaicity (°)	0.4	0.5
Resolution (Å)	40–2.5	40–2.9
Last resolution shell	2.6–2.5	2.9–2.8
Completeness (%)	96.7	99.9
Last resolution shell	98.9	100.0
$R_{\rm merge}$	0.066	0.107
Last resolution shell	0.28	0.52
$I/\sigma(I)$	15.1	13.0
Last resolution shell	3.6	2.7

† $R_{\rm merge}$ is defined as $\sum|I_h - \langle I_h\rangle|/\sum I_h$.

3. Results and discussion

X-ray diffraction quality crystals of MIP synthase were generated using the outlined conditions. Form I crystals grew to dimensions of 0.2 × 0.4 × 0.7 mm (Fig. 2*a*). Form II crystals have typical dimensions of 0.1 × 0.3 × 0.6 mm (Fig. 2*b*). Since spontaneous nucleation of both crystal forms was difficult, a microseeding protocol was developed for the form I crystals that consistently yields large single well diffracting crystals. In microseeding, two or three crystals from a previous crystallization were crushed in a 10 µl drop of a mother liquor (5% PEG 8000, 100 m*M* sodium acetate pH 4.5). These crushed crystals were then diluted in the mother liquor in a series ranging from 10^{-3} to 10^{-7}. The ideal dilution range for yielding the aforementioned crystals was $10^{-5.5}$ to $10^{-6.5}$. As a result, the microseeded crystals were better defined morphologically, with typical dimensions of 0.2 × 0.3 × 0.4 mm (Fig. 2*c*). Crystal form I diffracted to 2.5 Å with a data-set completeness of 96.7% and an $R_{\rm merge}$ of 6.6%. Form II crystals diffracted to 2.9 Å with a data-set completeness of 99.9%. The $R_{\rm merge}$ of form II is 10.7%. Both crystal forms are monoclinic, with form I belonging to space group *C*2 and form II belonging to $P2_1$. Unit-cell parameters and data statistics are given in Table 1 for the data sets collected from each form. The volume of the unit cell for the *C*2 form is consistent with a 120 kDa dimer in the asymmetric unit and a solvent content of 51% (Matthews, 1968). Similarly, the volume of the unit cell for the $P2_1$ form is consistent with a 240 kDa tetramer in the asymmetric unit and a solvent content of 50% (Matthews, 1968).

4. Conclusions

In conclusion, we have produced two reasonably well diffracting crystal forms of *myo*-inositol 1-phosphate synthase. A native data set has been collected from both forms. We have also collected data on several potential derivatives that are currently being analyzed. Recently, we crystallized selenomethionine-substituted protein for use in MAD phasing (Hendrickson *et al.*, 1990).

The determination of the high-resolution three-dimensional structure of the synthase should provide insight into the catalytic mechanism of the conversion of glucose 6-phosphate to *myo*-inositol. Elucidation of the structural details of the active site will be critical for the design of more potent inhibitors that may have therapeutic use in the treatment of manic depression.

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