myo-Inositol oxygenase: molecular cloning and expression of a unique enzyme that oxidizes myo-inositol and d-chiro-inositol

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myo-Inositol oxygenase (MIOX) catalyses the first committed step in the only pathway of myo-inositol catabolism, which occurs predominantly in the kidney. The enzyme is a non-haem iron enzyme that catalyses the ring cleavage of myo-inositol with the incorporation of a single atom of oxygen. A full-length cDNA was isolated from a pig kidney library with an open reading frame of 849 bp and a corresponding protein subunit molecular mass of 32.7 kDa. The cDNA was expressed in a bacterial pET expression system and an active recombinant MIOX was purified from bacterial lysates to electrophoretic homogeneity. The purified enzyme displayed the same catalytic properties as the native enzyme with $K_m$ and $k_{cat}$ values of 5.9 mM and 11 min$^{-1}$ respectively. The $pI$ was estimated to be 4.5. Preincubation with 1 mM Fe$^{2+}$ and 2 mM cysteine was essential for the enzyme’s activity. d-chiro-Inositol, a myo-inositol isomer, is a substrate for the recombinant MIOX with an estimated $K_m$ of 33.5 mM. Both myo-inositol and d-chiro-inositol have been implicated in the pathogenesis of diabetes. Thus an understanding of the regulation of MIOX expression clearly represents a potential window on the aetiology of diabetes as well as on the control of various intracellular phosphoinositides and key signalling pathways.

Key words: diabetes, d-glucuronate, kidney, mono-oxygenase, pig.

INTRODUCTION

myo-Inositol and its various biochemical derivatives are widely distributed in mammalian tissues, higher plants, fungi and some bacteria, where they are important in many aspects of cellular regulation including membrane structure, signal transduction and osmoregulation [1–3]. The first committed step in the metabolism of myo-inositol occurs predominantly in the kidney and involves the oxidative cleavage of the ring to give d-glucuronic acid, as shown in Figure 1 [4,5]. The d-glucuronate formed in animals by this mechanism is successively converted in subsequent steps to l-gulonate, 3-oxo-l-gulonate, L-xylulose, xylitol, D-xylulose and D-xylulose-5-phosphate, which then enters the pentose phosphate cycle [6].

myo-Inositol oxygenase (MIOX, EC 1.13.99.1) is of considerable interest physiologically because it catalyses the first committed step in the only pathway of myo-inositol catabolism. This reaction is therefore an important determinant of inositol levels in vivo. The enzyme was first reported in 1957 in rat kidney extracts [5] and was subsequently purified from rat kidney [7,8] and oat seedlings [9]. Because MIOX isolated from those sources was found to be very unstable, little information on its mechanism was determined and few detailed characteristics of the protein were ascertained. In the early 1980s we purified MIOX to electrophoretic homogeneity from pig kidney [10]. Of particular importance in developing the purification procedure was the finding that although the enzyme becomes catalytically less active during purification, it could be stabilized more easily by reconstitution with cysteine and ferrous iron [10]. This enabled us to study some of the basic physical and catalytic properties of the purified enzyme [11] as well as some of the preliminary mechanistic studies [12,13]. MIOX is an enzyme containing non-haem iron and catalyses a four-electron oxidation with the transfer of only one atom of oxygen into the product.

The catalytic mechanism of MIOX is unique among non-haem iron internal mono-oxygenases in biological systems and there are very few enzymes that catalyse an oxidative glycol cleavage reaction as MIOX does. Nevertheless, the purification of the enzyme from kidneys to electrophoretic homogeneity in adequate amounts for detailed mechanistic studies eluded and frustrated many investigators. Consequently, very little progress has been made on this enzyme in the intervening 20 years.

Any examination of physiological roles for myo-inositol and MIOX, the prime enzyme in its catabolism, requires a consideration of the possible impact of MIOX-modulated myo-inositol concentrations on signal transduction pathways, which involve inositol phosphates. As seen in the review by Majerus [2], the metabolism of inositol phosphates is quite complex and in mammalian systems is channelled through phosphatidylinositol as the common precursor of the important signalling molecules Ins(1,4,5)$P_3$ and Ins(1,4)$P_2$ as well as diacylglycerol. The production of intracellular myo-inositol-derived second messengers seems to be specifically involved in the control of cellular events such as the spatial and temporal organization of key signalling pathways, the rearrangement of the actin cytoskeleton and intracellular vesicle trafficking [14]. Although little linkage has been drawn between myo-inositol concentrations and phosphoinositide metabolism, there is some evidence of altered signal transduction and inositol phosphate metabolism in rat sciatic nerves during streptozotocin-induced diabetes[15]. Because levels of myo-inositol in diabetic animals are lower in the central nervous system, whereas the renal clearance of myo-inositol is...
enhanced, kidney MIOX levels might have a role in altered signal transduction. It is unclear how streptozotocin induces this effect and whether it precisely mimics diabetic neuropathy. However, it raises the prospect that modified myo-inositol levels might be important in understanding diabetic pathologies at the level of phosphoinositide signalling.

Although inositol catabolism in disease states such as diabetes has received little study, a large volume of work has been produced about altered levels of inositol in the diabetic state [16,17]. Of the physiological isomers of inositol, myo-inositol and \( \alpha \)-chiro-inositol are associated with diabetic pathologies [17,18]. Intracellular depletion of myo-inositol is associated with complications including diabetic nephropathy [19–21], retinopathy [22], neuropathy [23,24] and diabetic cataract [25,26]. Most importantly, the presence of MIOX in the mammalian lens has been demonstrated for the first time [25]. It is the first direct evidence that MIOX might have a role in the secondary pathologies of diabetes, and given the apparent similarity between the factors involved in neuropathy, nephropathy and retinopathy [27–29] it is conceivable that MIOX might well be involved in all three pathologies. \( \alpha \)-chiro-Inositol has been shown to be a component of an inositol phosphoglycan that mediates the action of insulin [30]. Infusion of the inositol phosphoglycan containing \( \alpha \)-chiro-inositol in streptozotocin diabetic rats correct the hyperglycaemic state [31,32]. The discovery of a common transporter protein with specificity for both myo-inositol and \( \alpha \)-chiro-inositol, which is inhibited by glucose, supports the increased clearance of both inositol isomers during hyperglycaemia [33].

Thus myo-inositol and \( \alpha \)-chiro-inositol seem to have a major role in the pathogenesis of diabetes and its related complications of nephropathy, retinopathy, neuropathy and cataract. An understanding of the major mechanism for the control of inositol metabolism at the level of MIOX might therefore open new windows to diabetic therapy. Here we report the cloning and expression of MIOX, which also exhibits significant enzyme activity towards \( \alpha \)-chiro-inositol. Overexpression of this enzyme would allow us to undertake a more detailed understanding of the structure and function of this important protein.

**EXPERIMENTAL**

**Pig kidney MIOX purification**

The pig kidney MIOX purification protocol was as reported previously from our laboratory [10] with the following modifications. Immediately after animals were killed in local slaughterhouses, kidneys were collected and placed on ice. The tissue was minced, washed with ice-cold water and homogenized in a Waring blender in 25 mM sodium acetate buffer, pH 6.0, containing 1 mM GSH and 1 mM PMSF. All subsequent purification steps were performed with the 25 mM sodium acetate buffer, pH 6.0 (standard buffer). Crude homogenate was fractionated with \( (NH_4)_2SO_4 \) and the pellet from the 35–60 % satd \( (NH_4)_2SO_4 \) fraction was collected, resuspended and dialysed against the standard buffer for further purification. This fraction was then subjected to anion-exchange chromatography on a DE-52 column; active fractions were pooled and concentrated. The MIOX activity pool was then applied to a Sephacryl-200 column for size-exclusion chromatography. Active fractions were collected, pooled, concentrated and subjected to chromatography on a phenyl-(hydrophobic interaction chromatography) (HIC) HPLC column.

**Partial protein sequencing**

The HIC-purified MIOX was separated by SDS/PAGE; 35 \( \mu \)g was blotted on a 0.2 \( \mu \)m PVDF membrane (Bio-Rad Laboratories, Hercules, CA, U.S.A.) in accordance with the manufacturer’s instructions. The major 33 kDa band, which was determined as MIOX by several criteria, was excised from the membrane and submitted for amino acid sequencing to the Columbia University Protein Chemistry Core Facility (New York, NY, U.S.A.). The N-terminus was blocked, so two internal amino acid sequences were recovered. The purified protein was also used to prepare monoclonal antibodies in mice at The Pennsylvania State University Life Sciences Consortium Hybridoma Facility (University Park, PA, U.S.A.).

**cDNA library construction**

A pig kidney cDNA library was constructed with a SMART cDNA library construction kit from Clontech Laboratories (Palo Alto, CA, U.S.A.) in accordance with the manufacturer’s instructions. In brief, mRNA from pig kidney was reverse transcribed with a poly(dT) primer and anchor primer. The single-stranded cDNA was amplified by PCR, digested and inserted into a TriplEx2 vector. The vector was packaged with Gigapack Gold packaging extract (Stratagene, La Jolla, CA, U.S.A.) and the library was amplified in accordance with the manufacturer’s indications.

**PCR cloning of MIOX**

Internal primer sequences were generated on the basis of known peptide sequences for PCR cloning from the pig kidney cDNA library. The sense primer (‘5’-CAGACAGTGGACTTCGT-CAGGA-3’) and anti-sense primer (‘5’-GTGCCAGGGGTGAAGGAGTGGAAC-3’) generated a product 531 bp in length. This amplimer was gel-purified and cloned into pGEM-T Easy vector (Promega, Madison, WI, U.S.A.) and sequenced at the
Purification of rMIOX

The frozen bacterial pellet was thawed on ice. All subsequent steps were performed at 4 °C unless otherwise mentioned. The pellet was ground in a mortar with glass beads (450–600 μm diameter; Sigma Chemical Co., St Louis, MO, U.S.A.) in 3 ml of standard buffer plus bacterial protease inhibitor cocktail. The lysate was transferred to a tube and the mortar was washed twice with standard buffer. The column was washed with 40 ml of standard buffer plus bacterial protease inhibitor cocktail. The dialysed lysate was transferred to a tube and the mortar was washed twice with standard buffer; washings were collected at 12500 rev./min for 11 h, 17000 rev./min for 17 h and 50000 rev./min (Beckman An60Ti) for 19 h at 4 °C. The scanning absorption detection system was adjusted to measure solution absorbance at 250 and 280 nm against appropriate blanks. These results were collected at regular intervals until successive scans gave identical profiles, indicative of equilibrium. The experiment was repeated at all three protein concentrations in the presence of (1) myo-inositol (60 mM), (2) ferrous iron (Fe²⁺) (1 mM) and cysteine (2 mM) and (3) myo-inositol plus Fe²⁺/cysteine. The partial specific volume (ν₀) was considered to be 0.73. Data were analysed to determine the molecular mass with the program ORIGIN (Microcal Software, Northampton, MA, U.S.A.). The rMIOX was loaded on a Sephadryl 200 gel-filtration column as described above. The purified rMIOX thus obtained was used for sedimentation equilibrium studies on a Beckman XL-1 analytical ultracentrifuge. Experiments were performed with 125 μl samples of the rMIOX at 0.2, 0.4 and 1.0 mg/ml concentrations in standard buffer. Fractions were pooled and concentrated to approx. 1 ml volume and loaded on a Sephacryl 200 gel-filtration column (212 ml bed volume; 1.5 cm × 120 cm column). Fractions of 2.3 ml were collected, then assayed for MIOX activity and analysed by SDS/PAGE. Fractions with MIOX activity were pooled and concentrated.

Western immunoblot analysis

The purified rMIOX was subjected to SDS/PAGE [12.5 % (w/v) gel] and transferred to a nitrocellulose membrane (pore size 0.2 μm). The membrane was immersed in a blocking solution of TBS-T buffer [0.05 % Tween 20/0.1 M Tris/HCl/0.15 M NaCl (pH 7.5)] containing 5 % (w/v) BSA. This was followed by successive incubations overnight at 4 °C with hydridoma cell supernatant and at 37 °C for 60 min with anti-mouse IgM conjugated with horseradish peroxidase (dilution 1:2500). The blot was washed three times in TBS-T and developed by the enhanced chemiluminescence detection method (Pierce Chemical Co., Rockford, IL, U.S.A.).

MIOX assay

Enzyme activities were determined by an orcinol assay system as described previously [10]. Kinetic parameters for myo-inositol and d-chiro-inositol were determined by the same assay method. Activity measurements were taken for myo-inositol over a concentration range of 0.5–50 mM, and for d-chiro-inositol from 5–300 mM. Kₘ and kcat values were calculated by regression analysis from Lineweaver–Burk plots.

Characterization of rMIOX

The molecular mass of the recombinant protein was investigated first. In addition to SDS/PAGE, a sample was submitted for electrospray MS determination at the Penn State Intercollegiate Mass Spectrometry Center (University Park, PA, U.S.A.). Also, the rMIOX was subjected to gel-filtration chromatography on a Sephacryl 200 column as described above. The purified rMIOX was subjected to SDS/PAGE [12.5 % (w/v) gel] and transferred to a nitrocellulose membrane (pore size 0.2 μm). The membrane was immersed in a blocking solution of TBS-T buffer [0.05 % Tween 20/0.1 M Tris/HCl/0.15 M NaCl (pH 7.5)] containing 5 % (w/v) BSA. This was followed by successive incubations overnight at 4 °C with hydridoma cell supernatant and at 37 °C for 60 min with anti-mouse IgM conjugated with horseradish peroxidase (dilution 1:2500). The blot was washed three times in TBS-T and developed by the enhanced chemiluminescence detection method (Pierce Chemical Co., Rockford, IL, U.S.A.).

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RESULTS

Purification of MIOX from pig kidneys

To obtain internal protein sequences on which to base PCR primer sequences for cloning, we purified pig kidney MIOX as described previously [10]. The yield of pure MIOX from 400 g of fresh tissue was 5.5 mg. HIC, which is a modification of the previously published procedure [10], was employed to purify MIOX, which was then used for amino acid sequencing (boxed area in Figure 2A). The purified MIOX consistently displayed one predominant band with a molecular mass of approx. 33 kDa (Figure 2B) and had a specific activity of 1250 nmol/min per mg.

Figure 2  Purification of native MIOX

(A) Phenyl-HIC purification of the gel filtration pool of MIOX activity. The activity pool from S-200 gel filtration was concentrated by ultrafiltration on an Amicon YM-10 membrane and filtered through a 0.2 mm syringe filter containing the indicated salt gradient (dotted line). The box denotes the range of eluate collected from several HPLC runs and combined as the 'HIC activity pool.' (B) SDS/PAGE analysis of eluate fractions from the HIC purification step. Right lane: MIOX preparation after HIC purification step was examined on an SDS/PAGE gel with Coomassie Blue staining for proteins. The amount of pig MIOX loaded was 4 μg. Left lane: molecular mass markers (molecular masses indicated in kDa at the left).

Figure 3  Open reading frame and alignment of deduced primary sequence of pig MIOX with hypothetical protein sequences

(A) The nucleotide sequence of the open reading frame of pig kidney MIOX and its deduced amino acid sequence are shown. A cysteine residue unique with regard to reported sequences in other species is indicated in bold. (B) Alignment of the amino acid sequences of pig MIOX and the sequences reported by Yang et al. [34]. Accession numbers are as follows: rat, AF197128; mouse, AF197127; human, AF197129; pig, AF401311.
N-terminus of the protein sequence. Amino acids (VDL...those of hypothetical submissions by Yang et al. was found in the (Figure 3B). One major difference between our sequence and rat and mouse sequences and 96% position in rat, mouse and human was missing from the pig mouse and human. The histidine residue present at the 49th position in rat, mouse and human. There were a total of ten histidine residues in the pig sequence, nine of which were common to those reported for the hypothetical sequences in rat, mouse and human. The histidine residue present at the C-terminal end (Figure 3B). The hypothetical sequences from other species had a conserved serine at this position. There were a total 6 cysteine residues conserved at positions 147, 156, 182, 234, 257 and 278. The pig sequence also had cysteine residues at all these positions except at 257 where it was glycine instead of cysteine. The sixth cysteine residue in pig had cysteine residues at all these positions except at 257 where it was tryptophan. Instead, the tenth histidine residue in pig was present at position 59. Interestingly, the human hypothetical sequence also had an additional histidine residue at this position, whereas the rat and mouse hypothetical sequences had arginine (Figure 3B).

Cloning of MIOX cDNA

A cDNA was PCR amplified from the pig kidney library containing the open reading frame of 849 bp (Figure 3A), as well as 5' and 3' untranslated region sequences totalling approx. 1.1 kb. Northern blot analysis indicated that the transcript was approx. 1.6 kb in length. A BLAST search conducted with National Center for Biotechnology Information online software revealed that the sequence was unique, with the only matches to sequences of hypothetical cDNA submissions (accession numbers AF197127, AF197129, AF197128) by Yang et al. [34], and a partial match to Pinus radiata sequence (accession number AF049069). The degree of similarity was approx. 86–89% to the rat and mouse sequences and 96% to the human sequence (Figure 3B). One major difference between our sequence and those of hypothetical submissions by Yang et al. was found in the N-terminus of the protein sequence. Amino acids (VDL/V; single-letter amino acid codes) found in the rat, mouse and human hypothetical sequences after the N-terminal methionine were missing from the pig sequence. Furthermore, the NADPH-binding motif sequence (MAKS) found in mouse and rat was also missing from the pig MIOX sequence, especially the essential serine residue. Instead, the pig sequence contained AAKD, which is similar to the human sequence (VAKD). The hypothetical sequences had six cysteine residues conserved at positions 147, 156, 182, 234, 257 and 278. The pig sequence also had cysteine residues at all these positions except at 257 where it was glycine instead of cysteine. The sixth cysteine residue in pig was present as the penultimate amino acid at the C-terminal end (Figure 3B). The hypothetical sequences from other species had a conserved serine at this position. There were a total of ten histidine residues in the pig sequence, nine of which were common to those reported for the hypothetical sequences in rat, mouse and human. The histidine residue present at the 49th position in rat, mouse and human was missing from the pig sequence, where it was tryptophan. Instead, the tenth histidine residue in pig was present at position 59. Interestingly, the human hypothetical sequence also had an additional histidine residue at this position, whereas the rat and mouse hypothetical sequences had arginine (Figure 3B).

Expression and purification of rMIOX

The pET17b/MIOX construct was transformed into BL21(DE3) cells and significant MIOX expression was observed after 4 h of induction with IPTG by SDS/PAGE analysis (Figure 4A, lane 3). Owing to interference in the orcinol assay at very low levels, detergents could not be used in the lysis and purification procedures for rMIOX. Instead, steps were performed in the standard buffer. As can be seen in the purification table (Table 1) and SDS/PAGE (Figure 4B, lane 5), the final rMIOX preparation was more than 95% pure, with a specific activity higher than that of the native enzyme. However, the yield of active MIOX in soluble fraction was relatively low, which seems to have been due to a large percentage of the rMIOX going into the insoluble inclusion bodies. Total cell-free extracts prepared in the presence of detergent showed that the amount of 33 kDa protein produced by BL21(DE3) cells induced with IPTG was indeed substantial (more than 10 mg/l; see Figure 4A, lane 3). Attempts were made to isolate the rMIOX from the purified inclusion bodies; however, the protein was inactive. Experiments designed to refold it were unsuccessful (results not shown). Supplementing the LB medium with 20 mM myo-inositol improved yields 2–3-fold in the soluble fraction. Media conditions for higher expression in the soluble fraction are being examined.

Characterization of rMIOX

The subunit molecular mass based on the translated amino acid sequence for the rMIOX cDNA was calculated to be 32.7 kDa. This was checked by SDS/PAGE (Figure 4B, lane 5) and electrospray MS. Results from MS indicated the molecular mass to be 32.663 kDa. In addition, gel-permeation chromatography on Sephacryl 200 was used to calculate the molecular mass of the rMIOX, which was found to co-elute with bovine erythrocyte
Table 1  Summary of the purification of recombinant MIOX from *E. coli*

The enzyme was purified from 2 litres of LB medium induced with 1 mM IPTG for 4 h as described in the Experimental section.

<table>
<thead>
<tr>
<th>Step</th>
<th>Total protein (mg)</th>
<th>Total activity (nmol/min)</th>
<th>Specific activity (nmol/min per mg)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude lysate</td>
<td>57.0</td>
<td>2268</td>
<td>40</td>
<td>100</td>
</tr>
<tr>
<td>35–60%-satd (NH₄)₂SO₄ fraction</td>
<td>18.0</td>
<td>2449</td>
<td>134</td>
<td>108</td>
</tr>
<tr>
<td>DE-52 column chromatography</td>
<td>5.0</td>
<td>1735</td>
<td>359</td>
<td>76</td>
</tr>
<tr>
<td>Sephacryl 200 column chromatography</td>
<td>0.8</td>
<td>1295</td>
<td>1546</td>
<td>57</td>
</tr>
</tbody>
</table>

Figure 5  Substrate kinetics of the oxidation of *myo*-inositol by rMIOX

Left panel: the concentration of *myo*-inositol (MI) was varied from 0.5 to 50 mM and the product, *d*-glucuronate, was assayed as described in the Experimental section. Right panel: Lineweaver–Burk plot.

superoxide dismutase (molecular mass 32.5 kDa). Western immunoblot analysis with the monoclonal antibodies prepared against the native pig MIOX confirmed the identity of expressed rMIOX (Figure 4C). The subunit molecular mass was confirmed by sedimentation equilibrium analysis. In addition, this analysis revealed that rMIOX did not dimerize on the addition of *myo*-inositol or *myo*-inositol plus Fe²⁺/cysteine to the enzyme. This is in contrast with the substrate-dependent oligomerization reported previously [35] for the rat kidney native MIOX.

An isoelectric focusing gel was run with two sets of standards (Sigma). The pI of MIOX was found to be 4.5 (results not shown), in agreement with the earlier studies on native MIOX from our group [10,11]. The enzymic activity, as determined by orcinol assay, was found to be heat labile and dependent on *myo*-inositol. As described previously by our group, the activity was also dependent on activation by Fe²⁺ and cysteine. This was accomplished by incubating the recombinant protein with 1 mM ferrous ammonium sulphate and 2 mM l-cysteine for 7 min before the addition of substrate. Other thiol compounds such as GSH, 2-mercaptoethanol and lipoic acid were tested and none were found to replace cysteine in the reaction, nor were they inhibitory. Kinetic parameters of rMIOX were in agreement with those reported previously [10,11]. The *Kₘ* for *myo*-inositol was 5.9 mM and the *kₐₙ* was 11 min⁻¹ (Figure 5). The affinity for *d*-chiro-inositol was less than that of *myo*-inositol; the *Kₘ* and *kₐₙ* for *d*-chiro-inositol were estimated to be 33.5 mM and 2.3 min⁻¹ respectively (results not shown). However, the kinetic parameters for the latter substrate could not be calculated accurately because the product of the reaction and its orcinol conjugate have not been characterized.

Yang et al. [34] reported NADPH binding for the mouse hypothetical protein, which they named as aldehyde reductase 6. Since our protein had 86% similarity to the hypothetical mouse protein, we examined the binding of rMIOX to a Reactive Blue Agarose column. No binding was detected because the flow-through fractions contained more than 95% of the MIOX protein.

DISCUSSION

MIOX is of considerable interest physiologically because it catalyses the first committed step in the only pathway of *myo*-inositol catabolism and it occurs predominantly in the kidney. Clearly this reaction is an important determinant of the inositol levels *in vivo*. One metabolic disease in which the enzyme might be especially significant is diabetes mellitus. Diabetics excrete excessive amounts of inositol in their urine and it seems that in addition to glucose interference with inositol transport, decreased activity of MIOX might also be involved [36]. Among other effects, the alterations of tissue levels of inositol in diabetics are believed to contribute to diabetic neuropathy. More recently, even greater attention has been focused on inositol metabolism because it has become clear that various inositol derivatives, especially the trisphosphates, act as second messengers in various signal transduction pathways in mammalian cells. In addition, MIOX is of considerable interest owing to its unusual mono-
oxygenase mechanism in which the ring of myo-inositol is cleaved with the incorporation of one atom of oxygen [12]. There are very few enzymes that catalyse an oxidative glycol cleavage reaction in this way.

We have now isolated and sequenced a cDNA clone encoding MIOX from pig kidney and expressed the rMIOX protein in bacteria. The bacterial expression system produces reasonable amounts of soluble and active MIOX and the expression is further enhanced 3-fold on supplementation of the medium with myo-inositol. However, most MIOX is found in inclusion bodies and attempts to refold it have not been successful. The enzyme is a 32.7 kDa protein that shows no significant sequence similarity to any known proteins. However, pig MIOX cDNA sequence has 86–89 % similarity to the rat and mouse clones of Yang et al. [34], which were reported as possible members of the aldo–keto reductase (AKR) family of enzymes. Whereas the mouse clone product examined by Yang et al. bound strongly to NADPH, no such interaction was observed with the pig rMIOX in terms of binding to Blue Agarose. This seems likely to be due to a mutation of an essential serine residue in the NADPH-binding domain. The human clone also lacks the serine and has a similar sequence to that of the pig clone in this region (Figure 3B). Furthermore, as in other Fe–sulphur-cluster-containing proteins, the presence of conserved histidine and cysteine residues in MIOX might indicate a potential role for these residues in enzyme function.

As mentioned above, a rat clone for a hypothetical protein has been sequenced, the sequence of which is almost identical with the mouse sequence and very similar to that of pig MIOX [34]. Although the authors reported it as a protein of the aldehyde reductase (ALR) family, it is conceivable that it could be MIOX. However, if this hypothetical protein in rat is indeed MIOX, this needs to be reconciled with the work of Koller and colleagues [35,37,38], who have extensively characterized the rat enzyme and shown MIOX to be an oligomer of 17 kDa subunits. Thus there is a considerable discrepancy between the subunit molecular mass of the native enzyme from rat kidney (17 kDa) and the predicted value from the reported hypothetical rat transcript, which is approximately double the size [34]. Our results with pig rMIOX prove convincingly that the pig protein has a molecular mass of 32.7 kDa and, in contrast with Koller’s observations [35,37,38], does not undergo oligomerization in the presence of myo-inositol.

Previously we had reported that the native MIOX enzyme is probably found in a complex with the enzyme responsible for the second step of myo-inositol catabolism, namely glucuronate reductase [10], which is also known as aldehyde reductase or ALR1 (EC 1.1.1.2) [39]. It was subsequently demonstrated that glucuronate reductase prefers the acyclic form of glucuronate [40]. When glucuronate was replaced with stoichiometric equivalents of MIOX and myo-inositol in the glucuronate reductase assays, a greater consumption of NADPH was observed than an equivalent amount of free glucuronate, which is predominantly in the cyclic form. It is presumed that MIOX can transfer the acyclic glucuronate directly to the reductase in the complex [10]. Currently we are examining the interactions of MIOX with expressed pig kidney ALR1 in vitro with a yeast two-hybrid system.

Because myo-inositol homoeostasis is altered in diabetes mellitus, we are increasingly interested in the role of MIOX in this disease. Renal clearance of myo-inositol in diabetic patients and diabetic rats has been shown to increase up to severalfold relative to control subjects. At the same time, kidney MIOX activity levels have been observed to decrease an average of 4-fold relative to controls [36]. In contrast, recent data from Yang et al. [34] show that the message levels of the mouse hypothetical protein are increased in diabetes. Although altered MIOX activity has not been localized within the kidney, a report has shown significant differences in MIOX distribution between the cortex and medulla in perinatal rabbit kidney [27]. However, there is a large body of literature to indicate that tissue levels of myo-inositol are decreased significantly in response to hyperglycaemia caused by diabetes, along with an accumulation of sorbitol. This decrease in inositol levels is thought to be a major factor in diabetic complications [17]. Because of this conflicting evidence, the exact role of MIOX in diabetes remains unclear.

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