THE PENNSYLVANIA STATE UNIVERSITY

The Graduate School

Intercollege Graduate Program in Genetics

MYO-INOSITOL OXYGENASE: MOLECULAR ENZYMEOLOGY
AND TISSUE SPECIFIC EXPRESSION

A Thesis in

Genetics

by

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Submitted in Partial Fulfillment

of the Requirements

for the Degree of

Doctor of Philosophy

May 2002
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ABSTRACT

myo-Inositol (MI) and its various biochemical derivatives are very widely distributed in mammalian tissues, higher plants, fungi and some bacteria where they play an important role in many aspects of cellular regulation including membrane structure, signal transduction and osmoregulation (Holub, 1986; Majerus, 1992; Loewus and Loewus, 1983). The first committed step in the metabolism of MI occurs predominantly in the kidney and involves the oxidative cleavage of the ring to give D-glucuronic acid (Howard and Anderson, 1967; Charalampous and Lyras, 1957). This reaction is catalyzed by the enzyme myo-inositol oxygenase (EC 1.13.99.1, MIOX). In order to generate sufficient pure enzyme for mechanistic study, as well as to clarify the discrepancies of past research, a porcine MIOX clone was generated and expressed in a bacterial system. A full-length cDNA was isolated from a porcine 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 value was estimated to be 4.5. Preincubation with 1 mM Fe$^{2+}$ and 2 mM cysteine was essential for the enzyme activity. D-chiro-inositol, a myo-inositol isomer, is a substrate for the rMIOX with an estimated $K_m$ of 33.5 mM. Both myo-inositol and D-chiro-inositol have been implicated in the pathogenesis of diabetes.

Previously the native MIOX enzyme was reported to be likely found in a complex with the enzyme responsible for the second step of MI catabolism, i.e. glucuronate
reductase (Reddy et al., 1981a), which is also known as aldehyde reductase or ALR1 (EC 1.1.1.2) (De Jongh et al., 1987). The MIOX:ALR1 complex was partially purified and the activity examined. MIOX activity was present without reactivation with Fe/Cys, as is required with pure MIOX. When inositol is supplied to the complex as substrate, activity can be detected by the consumption of NADPH by the reductase. Adding similar concentrations of free glucuronate as that produced by the MIOX activity resulted in no detectable activity, indicating the ALR1 was trapping acyclic glucuronate from MIOX. However, no activity was detected with inositol as substrate when the aldose reductase inhibitor Sorbinil was added at 10µM. The same concentration inhibited pure ALR1 by 90% when glucuronate was the substrate. No inhibition of recombinant MIOX by Sorbinil was observed. These results suggest the possibility that aldose reductase inhibitor treatment for diabetes complications may have an impact on the inositol catabolic pathway.

In order to study the physiological relevance of the MIOX:ALR1 complex, the expression pattern of MIOX must be established. The expression pattern of MIOX in hog tissues was examined by Western blot, Northern blot, and RT-PCR methods. The predominant source of protein and mRNA was found in kidney. *In situ* hybridization further localized the MIOX to the kidney proximal tubule epithelial cells. However, protein was also detected by Western blot in retina tissue. To examine the lens, a human lens epithelial cell model, HLE-B3, was employed for protein and mRNA detection. Both MIOX protein and mRNA were detected in these cells. In human and mouse, *in situ* hybridization detected MIOX in the kidney. Sections of other human organs failed to detect MIOX. LLC-PK1 were tested as a model for the study of MIOX expression in
kidney. These cells expressed low levels of MIOX compared to kidney tissue, and the MIOX was unresponsive to inducers and hyperglycemia. It was concluded that an animal model would be necessary for future studies of MIOX in vivo.

In conclusion, this work represents a foundation for the future study of MIOX and its physiological relevance. The expression of MIOX is not confined to the kidney, but was also detected in retina and human lens epithelial cells. All of these tissues are subject to complications brought on by Diabetes Mellitus. Since MI metabolism is deranged in diabetic tissues, MIOX may play a major role in the pathogenesis of diabetic complications.
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<th>Description</th>
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<tbody>
<tr>
<td>ALR1</td>
<td>aldose reductase</td>
</tr>
<tr>
<td>ALR2</td>
<td>glucuronate reductase</td>
</tr>
<tr>
<td>ARI</td>
<td>aldose reductase inhibitor</td>
</tr>
<tr>
<td>Fe²⁺/Cys</td>
<td>ferrous iron-L-cysteine reactivation complex</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>HIC</td>
<td>hydrophobic interaction chromatography</td>
</tr>
<tr>
<td>IPG</td>
<td>inositol phosphoglycan</td>
</tr>
<tr>
<td>MI</td>
<td>myo-inositol</td>
</tr>
<tr>
<td>MIOX</td>
<td>myo-inositol oxygenase</td>
</tr>
<tr>
<td>MMO</td>
<td>2 O,C-methylene-myoinositol</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NIDDM</td>
<td>Non-insulin-dependent Diabetes Mellitus</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PERK</td>
<td>Endoplasmic reticulum transmembrane eIF2alpha kinase</td>
</tr>
<tr>
<td>PI</td>
<td>Phosphatidyl inositol</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
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<tr>
<td>---------</td>
<td>------------</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenyl methyl sulfonyl fluoride</td>
</tr>
<tr>
<td>RACE</td>
<td>rapid amplification of cDNA ends</td>
</tr>
<tr>
<td>RMIOX</td>
<td>recombinant myo-inositol oxygenase</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse-transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>UTR</td>
<td>untranslated region</td>
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</table>
Acknowledgements

I’d like to express my sincere appreciation to Dr. Channa Reddy for his patience and unwavering belief in my abilities. I would also like to especially thank the members of the Reddy lab, in particular Dr. Sandeep Prabhu, Dr. Jerry Thompson and Dr. Mary Lou Eskew for your advice, enduring friendship, and assistance. To all the graduate students of the Reddy lab, I wish you all the best in your careers. For their support from afar, I thank my family and friends. Finally, I wish to dedicate this thesis to my parents, whose love and support made all my accomplishments possible.
Chapter 1

Introduction

Inositols

The inositols are a group of hexahydroxycyclohexanes, consisting of a six-carbon ring with one hydroxyl group on each carbon. There are 8 main epimers possible for inositol, four of which have been identified physiologically. *myo*-Inositol (MI) (Figure 1) makes up the overwhelming majority of inositols found in living systems (Lewin et al., 1976). The other physiologically identified epimers include *scyllo*-, *epi*-, and *chiro*-inositol. *scyllo*-Inositol has been identified in many tissues, including brain and nerve (Sherman et al., 1968a; Sherman et al., 1968b). *chiro*-Inositol has been identified in the urine and plasma of humans (Kennington et al., 1990). The non-physiological epimers include L-*chiro*-, *muco*-, *neo*-, and *allo*-inositol.

All of the inositols form a chair conformation in the carbon ring. The structural differences are found in the orientation of the hydroxyl groups. As can be seen in Figure 1, MI has five of its hydroxyl groups in an equatorial orientation, in plane with the ring. The 2C hydroxyl is in an axial position, perpendicular to the ring. D-*chiro*-inositol shares the 2C axial hydroxyl with MI, and also has the 3C hydroxyl in an axial conformation, in the opposite face from the 2C group. *scyllo*-Inositol is distinguished by having all hydroxyl groups in the equatorial orientation, forming a flat ring.
Figure 1. The structures of myo-inositol and D-chiro-inositol
Biosynthesis of MI occurs in a variety of cell types and organisms, including mammals, plants, fungi, and bacteria (Loewus and Loewus, 1983). MI is synthesized from glucose-6-P through a circularization reaction. 1L-MI-1-P Synthase catalyzes this reaction through a myo-inosose-2 1-P intermediate. Following this step, the 1L-MI-1-P is converted to MI by the enzyme MI Monophosphatase.

**Physiological Functions of Inositols**

Inositols are utilized in cellular functions including osmotic homeostasis and cellular signaling. Many cell types can take up MI as an organic osmolyte, without perturbing cellular functions. This protective mechanism is mediated through the activity of a Na\(^+\)-MI cotransporter (Yamauchi et al., 1993;Kitamura et al., 1998;Nakanishi et al., 1996). Cells exposed to a hyperosmotic environment, such as in the kidney, are especially dependent on this system (85). MI is also of major significance to cell signaling, as it is the precursor to a wide range of phosphoinositides, which are responsible for a diverse set of signal pathways (Holub, 1986;Majerus, 1992). Free MI pools serve as substrate for the enzyme CDP-diacylglycerol: inositol phosphatidyltransferase (PI synthetase), which has been identified in microsomal fractions of rat brain and liver (Holub, 1986). PI can undergo additional phosphorylations to yield a wide variety of messenger molecules (PIP’s). These messengers are important in the temporal and spatial organization of cellular pathways and physical structures (Payrastre et al., 2001).
D-chiro-inositol was found to be the component of an inositol phosphoglycan (IPG) that mediates insulin signaling (Asplin et al., 1993). This IPG is required for the normal cellular response to insulin. Depletion of IPG has been associated with insulin resistance in tissues (Asplin et al., 1993; Kennington et al., 1990; Larner et al., 1998).

**Metabolism of MI and History of MIOX Research**

The first committed step in the metabolism of MI occurs predominantly in the kidney and involves the oxidative cleavage of the ring to give D-glucuronic acid as shown in Figure 2 (Howard and Anderson, 1967; Charalampous and Lyras, 1957; Charalampous and Lyras, 1957). Cleavage of the ring occurs between the 6C and 1C, involving a four-electron transfer with 1 atom of oxygen incorporated into the product. The D-glucuronate formed in animals by this mechanism is successively converted in subsequent steps to L-gulonate, 3-keto-L-gulonate, L-xylulose, xylitol, D-xylulose, and D-xylulose-5-phosphate, which then enters the pentose phosphate cycle. Studies in human pentosuric patients confirmed that this pathway is the only pathway of MI catabolism (Hankes et al., 1969).

*myo*-Inositol oxygenase (MIOX, EC 1.13.99.1) is of considerable interest physiologically because it catalyzes the first committed step in the only pathway of *myo*-inositol catabolism. Hence this reaction is an important determinant of the inositol levels in vivo. The enzyme was first reported in 1957 in rat kidney extracts (Charalampous and Lyras, 1957). Charalampous first identified an enzyme activity from rat kidney extracts that converts MI to D-glucuronic acid. The following year, the rat enzyme was purified
Figure 2. The inositol catabolism pathway
from rat kidney and identified as myo-inositol oxygenase (Charalampous, 1959). The enzyme was identified as a 68 kDa protein, containing non-heme iron, with high specificity for MI. Sulfhydryl groups were necessary for activity and the pH optimum was around pH 7. A notable property of MIOX was its rapid inactivation upon storage for any period of time. In 1960, Charalampous proposed a mechanism for the reaction catalyzed by MIOX (Charalampous, 1960). It was proposed that the cleavage of MI occurred between carbons 1 and 6. One atom of oxygen was incorporated into the 1C position in the carboxyl group of glucuronic acid. The reaction intermediate was proposed to be an enediol with the two hydrogen atoms reducing one atom of oxygen to form water. Beyond the initial characterization reported in these initial papers, the instability of the enzyme activity proved limiting.

In 1967, two studies were published that began to elucidate the physiological role of MIOX. First, Howard et al (Howard and Anderson, 1967) identified the kidney as the major site in the body for MI catabolism. It was found that nephrectomized rats failed to catabolize MI to CO₂. It was also estimated that the catabolic activity of MIOX in the kidney was approximately equal to the daily dietary intake of MI. This suggested that the role of MIOX in kidney was to maintain the plasma levels of MI that were produced by endogenous synthesis in tissues. The other significant report of that year involved the administration of an inositol analog, 2 O, C-methylene MI (MMO) to rats (Weinhold and Anderson, 1967). In the study, rats injected with MMO suffered kidney failure and died. Medullar tissue was damaged extensively, leading to renal failure. It was also discovered that MI uptake in kidneys was decreased. The kidney failure was attributed to MMO inactivation of MIOX, as the MIOX activity was significantly decreased. However,
Weinhold admittedly could not put forward a sufficient explanation for the link between MIOX inactivation and kidney failure.

Koller et al purified MIOX from a plant source in 1976 (Koller et al., 1976). Using an affinity technique, a range of enzymes with different inositol specificities was purified. The MIOX enzyme was identified as a 62 kDa protein that formed oligomers up to tetramers. A few years later the same group purified MIOX from rat kidney (Koller and Hoffmann-Ostenhof, 1979). Unlike Charalampous, their rat MIOX was identified as a 17 kDa protein that oligomerized (Koller and Hoffmann-Ostenhof, 1979) and the smallest active unit was a tetramer. Over the next decade, they continued to characterize rat MIOX. In 1984, an affinity purification procedure was described, in which the protein bound to a hexodialdose matrix by oligomerizing to tetramers (Koller and Koller, 1984). The oligomerization was found later to be dependent upon concentration and the presence of substrate and iron (Koller and Koller, 1990).

The most significant studies and characterization of MIOX were published in 1981 from Gordon Hamilton’s laboratory (Reddy et al., 1981a; Reddy et al., 1981b). MIOX was purified to near homogeneity from porcine kidney. The porcine MIOX was a protein of 66 kDa, and contained non-heme iron. Like previous studies in rat, the enzyme rapidly lost activity upon purification. One of the most significant findings was that the enzyme could be reactivated by incubation with ferrous iron and cysteine. This allowed a more extensive characterization of the activity, and wide ranges of compounds were characterized for activation and inhibitory properties (Reddy et al., 1981b). In later work, the mechanism was elucidated somewhat. Oxygen-18 tracer studies confirmed Charalampous’ proposal that one atom of oxygen is incorporated into the product at the
carboxyl group (Moskala et al., 1981). The enediol reaction mechanism proposed by Charalampous (scheme 3 in Figure 3) would make it possible for one atom of oxygen to also be incorporated into the aldehyde group, and one atom in the carboxylate or the solvent. Therefore this mechanism was ruled out in favor of two possible alternate schemes (schemes 1 and 2, Figure 3), one which included \textit{myo}-inosose 1 as the reaction intermediate, and the other scheme with a glucodialdehyde intermediate. The 18O2 studies could not rule out the possibility of either schemes 1 or 2 (Figure 3). However, a subsequent study identified L-\textit{myo}-inosose 1 as a potent inhibitor of MIOX, while glucodialdehyde did not inhibit the reaction significantly, indicating the likely reaction scheme is the first (Naber et al., 1986). Also of considerable interest was the finding that porcine MIOX is found in a 250 kDa complex with several proteins, including glucuronate reductase, which catalyzes the second step of inositol catabolism, reducing D-glucuronate to L-gulonate (Reddy et al., 1981a). Glucuronate reductase is the protein identified as aldehyde reductase (ALR1), which shares significant identity with aldose reductase (ALR2), the enzyme implicated in diabetic complications. The physiological significance of this complex has yet to be explored, but it has been shown that glucuronate reductase efficiently traps the D-glucuronate produced by MIOX (Naber and Hamilton, 1987). In solution, D-glucuronate is found predominantly in the cyclic form. Since the reductase requires acyclic glucuronate, it was proposed that MIOX could directly supply acyclic glucuronate to the reductase before it cyclized in solution. This indicated that some form of substrate tunneling is taking place between MIOX and glucuronate reductase in the native complex.
Beyond the studies just described, little study has been reported on MIOX in the last 20 years. One study involved the characterization of MIOX activity during the development of rabbit kidneys (Bry and Hallman, 1991). It was found that MIOX activity levels are extremely low during fetal development. After birth, activity levels slowly rise during development until activity reaches the maximum levels at adulthood. It was proposed that MIOX activity is kept low during development in order to maximize the plasma levels of MI, as high MI concentrations are needed for proper development. The increase in activity to adulthood mirrors the decreasing requirement for free MI as maturity is reached. This last study represents the state of MIOX research to date. For a summary of the characterization of MIOX by different groups, see Table 1. The only MIOX paper not mentioned thus far involves MIOX and diabetes, and will be covered in the next section.
Figure 3. Proposed reaction mechanisms for MIOX
Table 1. Summary of the reported properties of MIOX

<table>
<thead>
<tr>
<th>Source</th>
<th>Properties</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>Rat kidney</td>
<td>68 kDa</td>
<td>(Charalampous, 1959; Charalampous, 1960)</td>
</tr>
<tr>
<td></td>
<td>non-heme iron containing</td>
<td></td>
</tr>
<tr>
<td></td>
<td>very labile in oxygen</td>
<td></td>
</tr>
<tr>
<td>Oat Seedling</td>
<td>62 kDa</td>
<td>(Koller et al., 1976)</td>
</tr>
<tr>
<td></td>
<td>oligomerizes to tetramers</td>
<td></td>
</tr>
<tr>
<td>Rat kidney</td>
<td>17 kDa</td>
<td>(Koller and Hoffmann-Ostenvof, 1979; Koller and Koller, 1984; Koller and Koller, 1990)</td>
</tr>
<tr>
<td></td>
<td>oligomerizes, tetramer active form</td>
<td></td>
</tr>
<tr>
<td></td>
<td>affinity purification</td>
<td></td>
</tr>
<tr>
<td>Hog kidney</td>
<td>66 kDa</td>
<td>(Reddy et al., 1981a; Reddy et al., 1981b)</td>
</tr>
<tr>
<td></td>
<td>can be activated with ferrous iron after</td>
<td></td>
</tr>
<tr>
<td></td>
<td>purification</td>
<td></td>
</tr>
<tr>
<td></td>
<td>forms complex with glucuronate reductase and</td>
<td></td>
</tr>
<tr>
<td></td>
<td>other proteins</td>
<td></td>
</tr>
<tr>
<td>Rabbit kidney</td>
<td>Activity localized to kidney cortex</td>
<td>(Bry and Hallman, 1991)</td>
</tr>
<tr>
<td></td>
<td>Development stage determines activity</td>
<td></td>
</tr>
</tbody>
</table>
**Inositol Metabolism and Non-insulin-dependent Diabetes Mellitus (NIDDM)**

**myo-Inositol Oxygenase Activity During NIDDM**

Whiting et al (Whiting et al., 1979) performed the only study to date that directly examines MIOX during Diabetes Mellitus. The activity enzymes involved in inositol catabolism and inositol phosphate metabolism were examined. Rats were injected with streptozotocin to induce diabetes. After 12 weeks, the activities of the enzymes were examined. Diabetic kidney mass and protein was increased. It was found that MIOX activity in diabetic kidneys was decreased by about 75% compared to controls. Renal clearance of inositol was also increased considerably. It was noted by the author that high glucose concentrations in plasma inhibited inositol transport and probably accounted for some of the increased inositol clearance observed.

The first occurrence of MIOX activity identified outside the kidney was reported by Goode et al (Goode et al., 1996). All of the activities of MI to xylitol pathway (Figure 2) were identified in rat lens. Goode proposed that the presence of this pathway may be the source of the high levels of xylitol that accumulate in lenses that develop diabetic cataracts. However, no experiments were performed in diabetic lens to support the proposal.

**Inositol and Diabetic Complications**

For the past 20 years, an extensive body of research has accumulated about the metabolic changes that accompany the pathogenesis of the complications of Diabetes Mellitus. A specific group of tissues is affected by the hyperglycemic conditions caused
by diabetes, including kidney, retina, nerve and lens. These tissues undergo the same
general metabolic derangements, including sorbitol accumulation, MI depletion, and
altered Na⁺-K⁺ ATPase activity (Winegrad, 1987). The accumulation of sorbitol is due to
increased flux of glucose through the polyol pathway, which is catalyzed by ALR2
(Thomas et al., 1993). The accumulation of sorbitol causes a depletion of intracellular
MI, which leads to a cascade of metabolic derangements (Winegrad, 1987; Cohen et al.,
1990; Palmano et al., 1977; Thomas et al., 1993). Figure 4 shows a proposed scheme for
the changes in metabolism of diabetic tissues. While much of the research has centered
on the correction of the polyol pathway, the mechanism behind the MI depletion still
remains unknown.
Figure 4. Proposed cycle of deranged metabolic activity during diabetes (Greene and Lattimer, 1986)

A representation of metabolic changes in tissues that undergo complications of Diabetes Mellitus adapted from Greene and Lattimer, 1986.
**NIDDM and PKC, Na\(^+\)-K\(^+\) ATPase**

NIDDM is responsible for affecting cellular regulation downstream from inositol and inositol phospholipid regulation, in particular protein kinase C (PKC) and Na\(^+\)-K\(^+\) ATPase activity. The effect on these proteins and their activities varies by the specific tissue or cell type examined. PKC activity is affected differently in a variety of cells. In kidney, diabetic proximal tubular cells have been shown to have an increase in PKC activation (Gagnon et al., 1999), while the Na\(^+\)-K\(^+\) ATPase is increased by PKC activation. Another study refined the PKC observations, finding that PKC is activated 25% more by diabetes in the brush border side of proximal tubular cells, while cytosolic and basolateral PKC was unchanged (Hise and Mehta, 1988). There is contradictory evidence about the relationship of the PKC activation and ATPase activity, as Chen et al (Chen, 1992) determined that in mouse proximal tubules the ATPase activity was increased 50%, but the PKC associated fraction of activity was abolished in diabetic tubules. Other cell types in the kidney also exhibit changes in PKC activity. Glomerular cells have been shown to increase localization of PKC alpha, to the membrane during diabetes, without significant changes in activity (Babazono et al., 1998). The same study found that PKC in glomerular epithelial, endothelial, and mesangial cells all expressed PKC isoforms differently during diabetes. Increases in PKC activation in endothelial and mesangial cells during diabetes has also been attributed to increased de novo synthesis of the activator diacylglycerol from the high glucose content of cells (Derubertis and Craven, 1994).
Cells of the retina and lens also display individual patterns of PKC and Na\(^+\)-K\(^+\) ATPase activity. In the retina, PKC activation increases with a concomitant decrease in Na\(^+\)-K\(^+\) ATPase activity that could be reversed by the administration of antioxidants (Kowluru et al., 1999). Similar results were obtained in the retinal vasculature (Kowluru et al., 1998). In contrast, PKC isoform gamma is decreased in diabetic lens epithelial cells (Lewis et al., 2001), and can be recovered by treatment with aldose reductase inhibitors. It is clear that to discern a role for PKC and Na\(^+\)-K\(^+\) ATPase in the pathogenesis of diabetic complications, the organ, tissue, and cell type must be considered. The specific activities are regulated differently depending on the function of the cell. However, in nearly all cases, NIDDM and hyperglycemia do alter the activities of these proteins and should be considered integral to the derangement of cellular metabolism and pathogenesis of diabetic complications.

**Diabetic Nephropathy**

Several changes take place in diabetic kidneys, the time and severity of each is determined by how acute the hyperglycemia. Kidneys increase mass through increased protein expression and cellular hypertrophy within the first few days of hyperglycemia (Cortes et al., 1987). These effects have been attributed partly to an increase in sensitivity to growth signals (Wolf and Ziyadeh, 1999). The glomerulus is also affected. Increased rate of filtration and increased protein leakage into the filtrate have been reported (Castellino et al., 1990; Cohen, 1986). Throughout the kidney, expression of aldose reductase and accumulation of sorbitol are a common factor in diabetic
complications (Dunlop, 2000; Narayanan, 1993; Stribling et al., 1989). This accumulation leads to MI depletion in some kidney cells (Cohen et al., 1995; Pickard and Hawthorne, 1978). Lastly, the activity of Na\(^+-\)K\(^+\) ATPase activity is altered, especially in tubular segments (Finegold and Strychor, 1988; Raccah et al., 1998).

**Statement of the Problem**

*myme*-Inositol (MI), the dominant form of the physiological inositol isomers, is utilized in many tissues and cell types as a precursor for the synthesis of second messengers and also as an organic osmolyte. The first committed step in the only pathway of its metabolism is catalyzed by *myme*-inositol oxygenase (MIOX; EC 1.13.99.1), and 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 plays an especially significant role is NIDDM. Intracellular levels of MI have been shown to decrease in many tissues affected by complications of NIDDM, such as diabetic nephropathy, retinopathy and cataract. More recently, even greater attention has been focused on inositol metabolism because it has become clear that various inositol derivatives, especially the triphosphates, act as second messengers in various signal transduction pathways in mammalian cells. Therefore, the time seems propitious for a concerted effort to determine the factors that regulate the expression of this important enzyme as well as to elucidate mechanism and structure of MIOX. The proposed research is based on the premise that the MIOX may be responsible for decreases in MI levels during NIDDM. Therefore, an understanding of the function and regulation of MIOX is the first step to defining its potential role in the etiology of diabetic complications. *Our central
hypothesis is that alterations in MI metabolism associated with complications arising from diabetes mellitus are attributable to changes in the expression of MIOX.

The Specific Aims of the proposed research are:

1. The first aim is to isolate and sequence a cDNA clone encoding MIOX from porcine kidney and to over-express the MIOX protein.

2. The second specific aim is to examine the properties and activity of the native MIOX protein complex from hog kidney.

3. The third specific aim is to use the molecular probes and knowledge acquired in the first specific aim to begin a study of the physiological and pathological relevance of MIOX.
Chapter 2

myo-Inositol Oxygenase: Molecular Cloning and Expression of a Unique Enzyme that Oxidizes myo-Inositol and D-chiro-inositol

Introduction

myo-Inositol (MI) and its various biochemical derivatives are widely distributed in mammalian tissues, higher plants, fungi and some bacteria where they play an important role in many aspects of cellular regulation including membrane structure, signal transduction and osmoregulation (Holub, 1986; Majerus, 1992; Loewus and Loewus, 1983). The first committed step in the metabolism of MI occurs predominantly in the kidney and involves the oxidative cleavage of the ring to give D-glucuronic acid (Howard and Anderson, 1967; Charalampous and Lyras, 1957). The D-glucuronate formed in animals by this mechanism is successively converted in subsequent steps to L-gulonate, 3-keto-L-gulonate, L-xylulose, xylitol, D-xylulose, and D-xylulose-5-phosphate, which then enters the pentose phosphate cycle (Hankes et al., 1969).

myo-Inositol oxygenase (MIOX, EC 1.13.99.1) is of considerable interest physiologically because it catalyzes the first committed step in the only pathway of MI catabolism. Hence this reaction is an important determinant of the inositol levels in vivo. The enzyme was first reported in 1957 in rat kidney extracts (Charalampous and Lyras, 1957) and subsequently purified from rat kidney (Charalampous, 1959; Charalampous, 1960) and oat seedlings (Koller et al., 1976). Because MIOX isolated from those sources
was found to be very unstable, not much information on its mechanism or detailed characteristics of the protein was determined. In the early 80’s MIOX was purified to electrophoretic homogeneity from porcine kidney (Reddy et al., 1981a). Of particular importance in developing the purification procedure was the finding that although the enzyme becomes catalytically less active during purification, it could be more easily stabilized by reconstitution with cysteine and ferrous iron (Reddy et al., 1981a). This enabled the study of some of the basic physical and catalytic properties of the purified enzyme (Reddy et al., 1981b) as well as some of the preliminary mechanistic studies (Moskala et al., 1981; Hamilton et al., 1982). \textit{myo}-inositol oxygenase is a non-heme iron-containing enzyme, which catalyzes a 4-electron oxidation with the transfer of only one atom of oxygen into the product.

The catalytic mechanism of MIOX is unique among non-heme iron internal monooxygenases in biological systems and there are very few enzymes that catalyze an oxidative glycol cleavage reaction as this one does. Nevertheless, the purification of the enzyme from kidneys to electrophoretic homogeneity in adequate amounts for detailed mechanistic studies was found to be elusive and frustrating to many investigators. Consequently, very little progress has been made on this enzyme in the intervening 20 years.
Methods

Porcine Kidney MIOX Purification

The porcine kidney MIOX purification protocol was as reported previously from our laboratory (Reddy et al., 1981a) 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 ammonium sulfate and the 35-60% pellet collected, resuspended and dialyzed against the standard buffer for further purification. This fraction was then subjected to anion-exchange chromatography on a DE-52 column with active fractions 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-HIC HPLC column.

Partial Protein Sequencing

The HIC purified MIOX was separated on SDS-PAGE and 35 µg was blotted onto a 0.2 µm PVDF membrane (Bio-Rad Laboratories, Hercules, CA) as per manufacturer’s instructions. The major 33 kDa band, which was determined to be MIOX by several criteria, was excised from the membrane and submitted for amino acid sequencing to the Columbia University Protein Chemistry Core Facility. The N-terminus was blocked; therefore, 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.

cDNA Library Construction

A porcine kidney cDNA library was constructed using a SMART cDNA library construction kit from CLONTECH (Clontech Laboratories, Palo Alto, CA) as per manufacturer’s instructions. Briefly, mRNA from porcine kidney was reverse transcribed using a poly-dT primer and anchor primer. The single stranded cDNA was PCR amplified, digested and inserted into a λTriplEx2 vector. The vector was packaged with Gigapack Gold packaging extract (Stratagene, La Jolla, CA) and the library amplified as per manufacturer’s indications.

PCR Cloning of MIOX

Internal primer sequences were generated based on known peptide sequences for PCR cloning from the porcine kidney cDNA library. The sense primer (5’-CAGACAGTGGACTTCGTCAGGA-3’) and antisense primer (5’-GTGCCAGGGGTAGAAGGAGTGGAAC-3’) generated a product of 531 bp in length. This amplimer was gel-purified and cloned into pGEM-T Easy vector (Promega, Madison, WI) and sequenced at the Penn State Nucleic Acid Facility to compare the translated sequence with the peptide sequence obtained from tissue purified MIOX. The remaining cDNA sequence was amplified by 5’ and 3’ RACE. 5’RACE was performed with the internal antisense primer above and a sense primer (5’-
GAAGCGCGCCATTGTGTTGGT-3’) based on the λ TriplEx2 vector sequence. The 3’ RACE was performed using the sense internal primer from above with CDSIII primer (CLONTECH). All PCR amplifications were performed with proofreading Pfα polymerase enzyme (Gibco-BRL, Rockville, MD) and amplimers were confirmed by sequencing both DNA strands at least in duplicate.

**Expression of Recombinant MIOX (rMIOX)**

For bacterial expression of MIOX cDNA, an insert was constructed by PCR using porcine kidney cDNA library as template. The full-length coding region was amplified with an Nde I site included in the sense primer (5’-GGAATTCCATATGAAGGACCCAGACCCTTC-3’) and a BamH I site in the antisense primer (5’-CGGGATCCCGTCACCAGCACAGGACA-3’). This PCR product was purified from a low-melting agarose gel, digested with Nde I and BamH I, and ligated into pET17b vector (Novagen, Madison, WI), also digested with Nde I and BamH I. The pET17b construct was transformed into DH5α competent E.coli cells and PCR screened for the presence of full length MIOX cDNA and proper orientation. Positive clones were grown and purified by plasmid miniprep kit (Promega). The plasmid was sequenced in triplicate for proper reading frame and polymerase fidelity. For expression, the pET17b/MIOX construct was transformed into BL21(DE3) cells (Novagen) and again PCR screened for the presence of pET17b-MIOX. A positive colony was grown in LB/ampicillin media as a starter culture for 5 h. Two ml of starter culture was then inoculated into 2 L of LB/amp media supplemented with 20mM MI and grown at 37 °C until the optical density at 600 nm reached 0.6. The culture was induced with 1mM IPTG
for 4 h. The cells were pelleted by centrifugation at 10,000 x g and the pellet frozen at –80 °C until the purification of rMIOX.

**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, Sigma Chemical Co. St Louis, MO) in 3 ml of standard buffer (25mM sodium acetate, pH6.0) plus bacterial protease inhibitor cocktail. The lysate was transferred to a tube and the mortar washed twice with an additional 3 ml of standard buffer and washings were pooled. The pooled lysate was sonicated briefly. Cell debris was pelleted by centrifugation at 20,000 x g for 15 min. Ammonium sulfate was added to the supernatant at a concentration of 35% and stirred for 1 h. Following centrifugation at 20,000 x g, the concentration of ammonium sulfate in the supernatant was brought to 60% and centrifuged once more. The 60% pellet was recovered and dissolved in 1 ml standard buffer, followed by dialysis against 1,000 volumes of standard buffer overnight. The dialyzed pellet was loaded onto a DE-52 column (20 ml bed volume, 1.5 x 20 cm column) and pre-equilibrated with standard buffer overnight. The column was washed with 40 ml of buffer, until the A280 was less than 0.01. A linear gradient of 0-0.2 M KCl (100 ml each) in the standard buffer was used to elute DE-52 bound MIOX. Fractions were assayed for MIOX activity using an orcinol-based assay described previously (Reddy et al., 1981a) and also analyzed on SDS-PAGE. Active fractions were pooled and concentrated to ~ 1 ml volume and loaded onto a Sephacryl 200 gel filtration column (1.5 x 120 cm, 220 ml bed volume). Fractions of 2.3 ml were collected and assayed for
MIOX activity and analyzed on SDS-PAGE. Fractions exhibiting MIOX activity were pooled and concentrated.

**Western Immunoblot Analysis**

The purified rMIOX was electrophoresed on an SDS-PAGE (T=12.5%) and transferred to a nitrocellulose membrane (0.2 µm). The membrane was immersed in a blocking solution (TBS-T (0.05% Tween-20/0.1 M Tris/0.15 M NaCl, pH 7.5) containing 5 % BSA). This was followed by successive incubations with hybridoma cell supernatant overnight at 4 ºC and anti-mouse IgM conjugated with horseradish peroxidase (1:2500) at 37 ºC for 60 min. The blot was washed three times in TBS-T and developed with the enhanced chemiluminescence detection method (Pierce Chemical Co., Rockford, IL).

**myo-Inositol Oxygenase Assay**

Enzyme activities were determined by an orcinol assay system as previously described (Reddy et al., 1981a). Kinetic parameters for MI and D-chiro-inositol were determined by the same assay method. Activity measurements were taken for MI over a concentration range of 0.5 mM to 50 mM, and for D-chiro-inositol from 5 mM to 300 mM. \(K_m\) and \(k_{cat}\) values were calculated from the Lineweaver-Burk plots by regression analysis.

**Characterization of rMIOX**

The molecular weight of the recombinant protein was first investigated. In addition to SDS-PAGE, a sample was submitted for electrospray mass spectrometric determination.
at the Penn State Intercollegiate Mass Spectrometry Center (University Park, PA). Also, the rMIOX was subjected to gel filtration chromatography on a Sephacryl 200 column as described above. The purified rMIOX, thus obtained, was used for sedimentation equilibrium studies on a Beckman XL-1 analytical ultracentrifuge. Experiments were carried out with 125 µl samples of the rMIOX at 0.2 mg, 0.4 mg and 1.0 mg/ml concentration in standard buffer. Absorption versus radius scans over a radial distance increment of 0.001 cm were collected at 12,500 rpm for 5 h, 15,000 rpm for 11 h, 17,000 rpm for 17 h and 50,000 rpm for 19 h at 4 ºC. The scanning absorption detection system was adjusted to measure solution absorbance at 250 nm and 280 nm against appropriate blanks. These data 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 (i) MI (60 mM), (ii) Ferrous iron (Fe2+)/Cysteine (1 mM / 2 mM), and (iii) MI + Fe2+/Cys. Value for the partial specific volume (γ) was considered to be 0.73. Data were analyzed to determine the molecular weight with the program ORIGIN (Microcal, Northhampton, MA). The rMIOX was loaded onto a Reactive Blue 4 agarose column (bed volume, 4 ml) to determine whether porcine rMIOX displays affinity towards NAPDH. The column was washed with standard buffer and eluted with 1 mM NADPH. The flow through fraction and NADPH eluate were analyzed by SDS-PAGE.

Partial Purification of MIOX Complex

The porcine kidney MIOX purification protocol was as reported previously from our laboratory (Reddy et al., 1981a) with the following modifications. Immediately after
animals were killed in local slaughterhouses, kidneys were collected and placed on ice. Cortex tissue from the kidneys, 400 g, was dissected after the capsule and fat material was removed from the kidney. The tissue was minced, washed with ice-cold water and homogenized in a Waring blender in 25 mM sodium phosphate buffer, pH 8.0, containing protease inhibitor cocktail (Sigma). Crude homogenate was fractionated with ammonium sulfate and the 35-60% pellet collected, resuspended and dialyzed against the phosphate buffer for further purification. This fraction was then applied to a hydroxylapatite column (100 g hydroxylapatite, 6 x 20 cm, 300 ml bed volume) and the flow through collected until A280 was less than 0.1. The column was regenerated by washing with 2L of 25 mM Na phosphate buffer, pH 8.0 and 0.5 M KCl, followed by equilibration with phosphate buffer. The flow through was then applied once more to the hydroxylapatite column and the clear yellow flow through collected and concentrated to 50 ml. This fraction, referred to as the HA fraction, was then assayed for MIOX and ALR1 activities.

**Aldehyde Reductase 1/Glucuronate Reductase (ALR1) Purification from MIOX Complex**

A 20 ml aliquot of the hydroxylapatite-purified fraction obtained in the complex purification was subjected to DE-52 Anion exchange chromatography. First, the HA fraction was dialyzed against 400 volumes of 25 mM Tris-Cl buffer, pH8.0. This sample was then applied to a DE-52 column (100 ml bed volume) that was pre-equilibrated with 25 mM Tris-Cl buffer, pH8.0. The column wash was monitored and fractions containing protein were assayed for ALR1 activity. Active fractions were pooled and concentrated to a volume of 5 ml, then subjected to gel filtration chromatography on a Sephacryl-200
column (1.5 x 120 cm, 220 ml bed volume). Fractions of 2.3 ml were collected and assayed for ALR1 activity and analyzed on SDS-PAGE.

**ALR1 Assays**

ALR1 activity was determined spectrophotometrically by following the rate of oxidation of NADPH at 340 nm in the reduction of glucuronate to L-gulonate. The reaction mixture included 50 mM Na phosphate buffer, pH 7.2, 100 mM NADPH, 10 mM D-glucuronate, and 2-50 µg enzyme. For ARI inhibition studies, 10µM Sorbinil, obtained as a gift from Pfizer (Groton, CT), was included in assay mixture.

**Complex Activity**

Activity of the MIOX:ALR1 containing complex was measured by the ALR1 assay with 60 mM MI substituted for D-glucuronate. The rate of oxidation of NADPH was monitored over a ten minute time period, with readings taken every 5 seconds.

**Protein Kinase C (PKC) Modification of MIOX**

Radioactive phosphorylation experiments were performed to determine the ability of MIOX to serve as substrate for PKC. The reactions were performed in a buffer containing 20 mM Tris-Cl, pH 7.5, 5 mM MgCl₂, 0.2 mM CaCl₂, 10 µg/ml diolein, and 100 µg/ml phosphatidylserine. ³²P labeled dATP (50 µCi) was added and purified rMIOX (10 µg, 4µg, and 0.5 µg) was used as substrate. The reaction was started by addition of 1 µg PKC α/β mixture (Promega). Total reaction volume was 20 µl. Reactions were run at 30 ºC for 30 minutes. The reactions were then separated by SDS-
Autoradiographs were then taken by exposure of the gel to film at room temperature for 20 minutes.

The effects of phosphorylations on MIOX activity were performed identically to the labeling reaction, with cold dATP substituted. One microgram of rMIOX was added as substrate to each reaction. MIOX activity was then measured by orcinol assay as reported previously (Reddy et al., 1981a).

**Immunoprecipitation of MIOX complex**

MIOX:ALR1 complex was immunoprecipitated from hydroxylapatite purified complex fraction. MIOX primary antiserum (1-10 µl) was incubated 1 hour at 4 ºC with 1 mg of HA fraction protein in a total volume of 1ml PBS buffer (1x), pH7.5. Then 40 µg of protein A/G PLUS agarose (Santa Cruz Biotechnology) was added and incubated 2 hours with gentle shaking at 4 ºC. The samples were centrifuged at 1000 x g for 5 minutes and the supernatant discarded. The agarose pellet was washed 4 times with 1 ml PBS, centrifuged for 5 minutes, and the supernatant discarded. To the final pellet, 20 µl of SDS running buffer, and boiled 5 minutes. The samples were analyzed by SDS-PAGE and Coomassie stained.
Results

Purification of MIOX from Porcine Kidneys

In order to obtain internal protein sequences on which to base PCR primer sequences for cloning, the porcine kidney MIOX was purified as described previously (Reddy et al., 1981a) (Table 2). The yield of pure MIOX achieved from 400 g of fresh tissue was 5.5 mg. Hydrophobic interaction chromatography (HIC), which is a modification of the previously published procedure (Reddy et al., 1981a), was employed to purify MIOX, which was then used for amino acid sequencing (boxed area in Figure 5A). The purified MIOX consistently displayed one predominant band with a molecular mass of ~33 kDa (Figure 5B) and had a specific activity of 1250 nmoles/min/mg towards MI. The HIC purified MIOX preparation was used for obtaining internal peptide sequences, which were then used to generate primer sequences for PCR cloning from the porcine kidney cDNA library.

Cloning of MIOX cDNA

A cDNA was PCR amplified from the porcine kidney library containing the open reading frame of 849 bp (Figure 6), as well as 5’ and 3’ UTR sequence totaling about 1.1 kb including the poly-A tail. Northern blot analysis indicated the transcript was about 1.6 kb in length. A BLAST search conducted with National Center for Biotechnology Information online software revealed the sequence was unique, with the only matches to sequences of hypothetical cDNA submissions from mouse, human, and rat (accession numbers AF197127, AF197129, AF197128, respectively) by Yang et al (Yang et al.,
2000), and a partial match to Pinus radiata sequence (accession number AF049069). Other than these hypothetical protein sequences, there was no sequence identity with any known cDNA or protein. The degree of identity was found to be 86% with the rat and mouse sequences and 89% with the human sequence (Figure 7). 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) found in the rat, mouse, and human hypothetical sequences after the N-terminal methionine are missing in porcine sequence. Furthermore, the NADPH binding motif sequence (MAKS) found in mouse and rat is also missing in the porcine MIOX sequence, especially the essential serine residue. Instead, the porcine sequence contains AAKD, which is similar to the human sequence (VAKD). The hypothetical sequences have 6 cysteine residues conserved at positions 147, 156, 182, 234, 257 and 278. The porcine sequence also has cysteine residues at all these positions except at 257 where it is glycine instead of cysteine. The 6th cysteine residue in porcine sequence is present as the penultimate amino acid at the C-terminal end (Figure 7). The hypothetical sequences from other species have a conserved serine at this position. There are a total of 10 histidine residues in the porcine sequence, 9 of which are common with those reported for the hypothetical sequences in rat, mouse and human. The histidine present at the 49th position in rat, mouse and human is missing in the porcine sequence where it is a tryptophan. Instead, the 10th histidine in porcine is present at position 59. Interestingly, the human hypothetical sequence also has an additional histidine at this position, whereas the rat and mouse hypothetical sequences have arginine (Figure 7).
Figure 5 Purification of native MIOX

A. Phenyl-hydrophobic interaction chromatogram 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 prior to HPLC. Samples containing ~3 mg of protein in 1 M ammonium sulfate were chromatographed on a SigmaChrom phenyl-HIC HPLC column and eluted at 0.9 ml/min with standard buffer containing the indicated salt gradient. 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 for the HIC purification step. MIOX preparation after HIC purification step (second lane) was examined on SDS-PAGE gel with Coomassie Blue staining for proteins. Load amount of porcine MIOX was 4 µg. First lane corresponds to molecular weight marker.
Table 2. Summary of the purification of native MIOX from porcine kidney

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<th>Fraction</th>
<th>Total activity, (nmol/min)</th>
<th>Total protein, (mgs)</th>
<th>Specific activity, (nmol/min/mg)</th>
<th>Yield (%)</th>
<th>Fold Purification</th>
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<tr>
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<td>946.0</td>
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Table 1. Partial peptide sequences of BSA

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<tr>
<td>HAQF</td>
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Figure 6  Nucleotide and protein sequence of porcine MIOX

The Boxed sequences represent partial peptide sequences obtained from native porcine MIOX. The cysteine residue unique to the porcine MIOX sequence is shown in bold.
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</tbody>
</table>

**Figure 7** Sequence alignment of porcine MIOX and the sequences reported by Yang et al. (Yang et al., 2000)

The NADPH-binding motif found in the mouse sequence is indicated in bold. Accession numbers are as follows: rat, AF197128; mouse, AF197127; human, AF197129; pig, AF401311.
**Expression and Purification of rMIOX**

The pET17b/MIOX construct was transformed into BL21(DE3) cells and significant MIOX expression was observed after 4 h induction with IPTG by SDS-PAGE analysis (Figure 8A, lane 3). Due to interference in the orcinol assay at very low levels, detergents could not be used in the lysis and purification procedures for recombinant MIOX. Instead, steps were performed in the standard buffer. As can be seen in the purification table (Table 3) and SDS-PAGE (Figure 8B, lane 5), the final rMIOX preparation was greater than 95% pure with specific activity higher than the native enzyme. However, the yield of active MIOX in soluble fraction was relatively low, which appears to be due to a high percentage of the rMIOX going into the insoluble inclusion bodies. Total cell free extracts prepared in the presence of detergent show that the amount of 33-kDa protein produced by BL21(DE3) cells induced with IPTG is indeed substantial (>10 mg/L, see Figure 8A, lane3). Attempts were made to isolate the recombinant MIOX from the purified inclusion bodies; however the protein was inactive. Experiments designed to refold were not successful (data not shown). Supplementing the LB media with 20 mM MI has 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 weight based on the translated amino acid sequence for the rMIOX cDNA was calculated to be 32.7 kDa. This was further checked by SDS-PAGE (Figure 8B, lane 5) and electro spray mass spectrometry. Results from mass spectrometry indicated the molecular mass to be 32.663 kDa. In addition, gel-permeation
Table 3 Summary of the purification of recombinant MIOX from *E.coli*

The enzyme was purified from 2 L of LB media induced with 1mM IPTG for 4 h as described under “Experimental”. Total activity and specific activity are in nmoles/min and nmoles/min/mg, respectively.

<table>
<thead>
<tr>
<th>Step</th>
<th>Total Protein (mg)</th>
<th>Total Activity</th>
<th>Specific Activity</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% Ammonium Sulfate 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 8  SDS-PAGE of rMIOX at different stages of purification

A. SDS-PAGE of total cellular lysates of BL21(DE3) cells prepared using 2% SDS expressing rMIOX after induction with 1mM IPTG. Lane 1 is a 10kDa marker (Gibco-BRL); Lane 2 is the uninduced cells; Lane 3, after 4 h IPTG induction. B. SDS-PAGE of the expressed rMIOX purification is shown. Lane 1, 10kDa ladder; lane 2, crude cell lysate-supernatant of ground and sonicated cells; lane 3, 35-60% ammonium sulfate fraction; lane 4, DE-52 active pooled fractions; lane 5, S-200 gel filtration pooled active fractions. The load per lane is 4µg. C. Western immunoblot analysis of the purified rMIOX using monoclonal antibodies prepared against the native porcine MIOX.
chromatography on Sephacryl 200 was used to calculate the molecular mass of the rMIOX and the rMIOX was found to co-elute with bovine erythrocyte superoxide dismutase (Mr 32.5 kDa). Western immunoblot analysis using the monoclonal antibodies prepared against the native porcine MIOX confirmed the identity of expressed rMIOX (Figure 8C). The subunit molecular weight was further confirmed by sedimentation equilibrium analysis. Also, this analysis revealed that rMIOX did not dimerize upon addition of MI or MI and Fe$^{2+}$/Cys to the enzyme. This is in contrast to the substrate-dependent oligomerization reported previously by Koller et al for the rat kidney native MIOX (Koller and Koller, 1990).

An isoelectric focusing gel was run with two sets of standards (Sigma). The pI of MIOX was found to be 4.5 (data not shown), which is in agreement with the earlier studies on native MIOX from our laboratory (Reddy et al., 1981b; Reddy et al., 1981a). A pI of 4.9 was calculated from the protein sequence. The enzymatic activity, as determined by orcinol assay, was found to be heat labile and MI-dependent. As previously described by our group, the activity was also dependent on the activation by Fe$^{2+}$ and cysteine. This was accomplished by incubating the recombinant protein with 1 mM ferrous ammonium sulfate and 2 mM L-cysteine for 7 min prior to substrate addition. Other sulfhydryl compounds, such as GSH, β-mercaptoethanol, and lipoic acid, were tested and none were found to replace cysteine in the reaction, nor were they inhibitory. Kinetic parameters of rMIOX are in agreement with those previously reported (Reddy et al., 1981b; Reddy et al., 1981a). The $K_m$ for MI was 5.9 mM and the $k_{cat}$ was 11 min$^{-1}$ (Figure 9). Affinity for D-chiro-inositol was less than that of MI; the $K_m$ and $k_{cat}$ for D-chiro-inositol were estimated to be 33.5 mM and 2.3 min$^{-1}$, respectively (data not
shown). However, the kinetic parameters for the latter substrate could not be calculated accurately as the product of the reaction and its orcinol conjugate have not been characterized. Furthermore, the epimers L-*chiro*-inositol and *scyllo*-inositol were tested as substrates for MIOX. No activity was detected with either epimer.

Yang et al reported NADPH binding for the mouse hypothetical protein, which they have named aldehyde reductase 6 (Yang et al., 2000), but have not identified a an activity for the recombinant protein. Since our protein has an 86% homology to the hypothetical mouse protein, I examined the binding of rMIOX to a Reactive Blue Agarose column. No binding was detected as the flow through fractions contained greater than 95% of the MIOX protein.

**Enzyme Stability**

The effect of storage conditions on the stability of rMIOX was examined. Most preparations of rMIOX had specific activities after S-200 gel filtration between 1200-1400 nmole/min/mg, which is somewhat lower than expected based on the maximum activity obtained from native MIOX in some preparations. However, the specific activity was observed to gradually increase over the course of 10 days storage at 4 ºC to the maximum value obtained from native MIOX, 2000 units. This increase was accompanied by the accumulation of a small amount of precipitate, which usually did not affect the protein concentration, as determined by BCA assay. Enzyme that was stored at −20 ºC for 3 months and assayed immediately after thawing had gained over 200 specific activity to 2266. Surprisingly, storing this sample further at 4 ºC for 10 days caused a dramatic increase in specific activity to ~3800. The increase was only observed in the
sample stored at –20 ºC, while samples stored at 4 ºC and never frozen did not gain activity above 2000 specific activity. Refrigerated samples maintained a significant portion of their activity after prolonged storage, as 6 months at 4 ºC caused only a 33% loss of specific activity. A summary of the effects of storage condition of activity is found in Table 4.
Figure 9. Substrate kinetics of the oxidation of MI by rMIOX

The concentration of MI was varied from 0.5 to 50 mM and the product, D-glucuronate, was assayed mentioned in “Experimental”. *Inset:* Lineweaver-Burke plot
Table 4. Summary of Enzyme Stability Experiments

<table>
<thead>
<tr>
<th>Storage Condition</th>
<th>Specific Activity (nmole/min/mg)</th>
<th>Fold increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh preparation</td>
<td>1200</td>
<td>1</td>
</tr>
<tr>
<td>10 days 4°C</td>
<td>2031</td>
<td>1.7</td>
</tr>
<tr>
<td>3 months –20°C</td>
<td>2266</td>
<td>1.9</td>
</tr>
<tr>
<td>3 months –20°C, 10 days 4°C</td>
<td>3826</td>
<td>3.2</td>
</tr>
<tr>
<td>6 months 4°C</td>
<td>1367</td>
<td>1.1</td>
</tr>
<tr>
<td>7 months –20°C</td>
<td>1907</td>
<td>1.6</td>
</tr>
</tbody>
</table>
Complex Activity

The complex containing MIOX was partially purified from hog kidney. Due to the lability of the complex, purification beyond the hydroxylapatite step was not successful. Even weak anion exchangers would dissociate most of the ALR1 from the complex with MIOX. Since the HA fraction proved to most stable when highly concentrated, this fraction was used for further experimentation. The proportion of MIOX and ALR1 activity recovered in the HA fraction were similar, as seen in the purification table (Table 5). Immunoprecipitation was also performed to determine the number of proteins present in the complex. The complex was found to contain MIOX, a 37 kDa band identified as ALR1, and two other bands of ~39 – 43 kDa (Figure 10), which is in agreement with Reddy et al. (Reddy et al., 1981a).

To examine the ability of ALR1 to trap the glucuronate produced by MIOX, the rate of oxidation of NADPH was monitored with MI as substrate. Figure 11 shows the relative rates of NADPH oxidation by the complex with and without 60mM MI. The velocity was calculated to be 1.01 µmole/min in a reaction containing 470 µg protein. Addition of 10 µM Sorbinil abolished all detectable activity. Also, 10 µM Sorbinil inhibited purified ALR1 by 90% with glucuronate as substrate. The same result was obtained when glucuronate was the substrate for HA fraction complex protein. No detectable effect on MIOX activity in purified, ferrous iron activated MIOX was observed.
Figure 10. Immunoprecipitation of MIOX:ALR1 complex

Partially purified HA fraction complex was immunoprecipitated with MIOX polyclonal antibody. Lane 1, MW marker; Lane 2, pure rMIOX; Lane 3, immunoprecipitated from 5 µg MIOX antibody; Lane 4, immunoprecipitated from 3 µg MIOX antibody. Precipitations were performed from 500 µg HA fraction protein.
PKC Modification of rMIOX

Sequence analysis of porcine MIOX performed with online software from the Center for Biological Sequence Analysis (Technical University of Denmark; http://www.cbs.dtu.dk/) indicated that MIOX sequence contained multiple potential phosphorylation sites. Since PKC plays a prominent role in the etiology of diabetic complications, it was of interest to examine the effect of PKC on MIOX activity. Experiments were also performed to determine the ability of rMIOX to serve as substrate for PKC α/β. Radioactive phosphorylation experiments identified that indeed, rMIOX is phosphorylated by PKC in vitro. No labeling was observed in the 33 kDa band when either $^{32}$P dATP or PKC were omitted (Figure 12). The phosphorylations of rMIOX affected activity of the activated enzyme. Phosphorylated MIOX exhibited a 55% decrease in activity compared to controls.
Figure 11. Oxidation of NADPH by partially purified MIOX native complex

Native MIOX complex was assayed for activity in the presence of NADPH and MI. The rate of decrease in absorbance at 340nm was monitored over 10 minutes in the presence or absence of 60mM MI. $V = 1 \text{ nmole/min.}$
Figure 12. Phosphorylation of rMIOX by PKC

rMIOX was incubated with PKC in the presence of $^{32}$P labeled dATP. Lane 1, control, no PKC or dATP; Lane 2, control, PKC + dATP, no rMIOX; Lane 3, rMIOX + dATP, no PKC; Lane 4, 10µg rMIOX; Lane 5, 5µg rMIOX; Lane 6, 0.4µg rMIOX. The 85 kDa band represents PKC with $^{32}$P-labeled dATP bound. Labeled rMIOX is the 33 kDa band.
Table 5. Summary of the purification of native MIOX complex from hog kidney

<table>
<thead>
<tr>
<th>Step</th>
<th>total protein (mg)</th>
<th>MIOX Specific Activity (per mg)</th>
<th>MIOX Yield %</th>
<th>ALR1 Specific Activity (per mg)</th>
<th>ALR1 Yield %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytosol</td>
<td>25091</td>
<td>0.6</td>
<td>100%</td>
<td>0.08</td>
<td>100%</td>
</tr>
<tr>
<td>Ammonium Sulfate Cut</td>
<td>8916</td>
<td>2.1</td>
<td>120%</td>
<td>0.17</td>
<td>76%</td>
</tr>
<tr>
<td>Hydroxylapatite</td>
<td>2366</td>
<td>3.2</td>
<td>48%</td>
<td>0.55</td>
<td>65%</td>
</tr>
</tbody>
</table>
Discussion

*myo*-Inositol oxygenase is of considerable interest physiologically because it catalyzes the first committed step in the only pathway of MI 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 may play an especially significant role is NIDDM. Diabetics excrete excessive amounts of inositol in their urine and it appears that in addition to glucose interference of inositol transport, decreased activity of MIOX may be involved as well (Whiting et al., 1979). Among other effects, the alterations of tissue levels of inositol in diabetics is 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 triphosphates, act as second messengers in various signal transduction pathways in mammalian cells. In addition, MIOX is of considerable interest due to its relatively unique monooxygenase mechanism in which the ring of MI is cleaved with the incorporation of one atom of oxygen (Moskala et al., 1981). There are few enzymes that catalyze an oxidative glycol cleavage reaction.

The cDNA clone encoding MIOX from porcine kidney was isolated, sequenced and the rMIOX protein was expressed in bacteria. The bacterial expression system produces reasonable amounts of soluble and active MIOX and the expression is further enhanced three fold upon supplementing the medium with MI. However, the majority of MIOX is found in inclusion bodies, and attempts to refold have not been successful. The enzyme is a 32.7-kDa protein that shows no significant sequence homology to any
characterized proteins. However, porcine MIOX cDNA sequence has 86% homology to the hypothetical rat and mouse clones of Yang et al (Yang et al., 2000), which were reported as possible members of the aldo-keto reductase (AKR) family of enzymes. While the mouse clone product examined by Yang et al bound strongly to NADPH, no such interaction was observed with the porcine rMIOX in terms of binding to Blue Agarose. This appears likely to be due to a mutation of an essential serine residue in the NADPH binding domain. The human clone is also missing the serine and has a similar sequence to the porcine clone in this region (Figure 7). Furthermore, as in the case of other Fe-sulfur cluster containing proteins, the presence of conserved histidines and cysteines in MIOX might indicate a potential role these residues may play in enzyme function.

As mentioned above, a rat clone for a hypothetical protein has been sequenced, which is almost identical to the mouse sequence and very similar to our sequence (Yang et al., 2000). Although, they have reported it as a protein of 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 (Koller and Koller, 1990; Koller and Hoffmann-Ostenhof, 1979; Koller and Koller, 1984), who has 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 weight 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 (Yang et al., 2000). These results with porcine rMIOX convincingly prove that the porcine protein has a molecular mass of 32.7 kDa and, in contrast to Koller’s observations (Koller and Koller, 1990; Koller and
Hoffmann-Ostenhof, 1979; Koller and Koller, 1984), does not undergo oligomerization in the presence of MI.

Also of interest is the ability of PKC to phosphorylate MIOX *in vitro*. Altered levels of PKC activation and activity are associated with diabetic complications (Gagnon et al., 1999; Lewis et al., 2001). It was observed that PKC phosphorylation significantly inhibited MIOX activity. The significance of these findings must be considered, however, on a specific tissue basis. Some reports indicate that PKC activity is depressed in certain tissues, while in other tissues it is increased. Depression of PKC activity could potentially increase overall MIOX activity if a smaller proportion of the MIOX is inhibited. The opposite would be true in tissues where PKC is reported increased. Further studies are required to determine the significance of these findings *in vivo* in relation to MIOX in complex with ALR1.

In this study the MIOX:ALR1 complex was partially purified, as further purification tends to degrade this labile protein association. The MIOX and ALR1 activities purified in comparable proportion in each step as seen in the purification table (Table 5). MIOX activity in this fraction was detectable without activation by Fe/Cys, indicating that the complex was largely intact. While Sorbinil was found to inhibit ALR1 activity, both in pure enzyme and in HA complex fractions, no effect was detected on MIOX activity. It is likely that treatment with Sorbinil for the complications of NIDDM not only corrects polyol pathway overactivity, but also inhibits glucuronate-xylulose pathway activity.

Since MI homeostasis is altered in NIDDM, it is interesting to study the role played by MIOX in this disease. Renal clearance of MI in diabetic patients and diabetic
rats has been shown to increase up to several folds relative to control subjects. At the same time, kidney MIOX activity levels decrease on an average of 4 fold relative to controls (Whiting et al., 1979). In contrast, recent data from Yang et al (Yang et al., 2000) shows that the message levels of the mouse hypothetical protein are increased by diabetes. While 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 (Bry and Hallman, 1991). However, there is a large body of literature, that indicates that tissue levels of MI are decreased significantly in response to hyperglycemia caused by diabetes, along with an accumulation of sorbitol. This decrease in inositol levels is thought to be a major factor in diabetic complications (Winegrad, 1987). Because of this conflicting evidence, the exact role of MIOX in diabetes remains unclear.
Chapter 3

Determination of the Tissue Specific Expression Pattern of MIOX

_in vivo_ and Attempts to Model a Cell Culture System

**Introduction**

Altered MI metabolism has been the hallmark of diabetic complications for more than two decades. In the major tissues where complications occur, including kidney, lens, retina, and nerve, MI is depleted (Winegrad, 1987). Depletion of MI has been proposed to lead to a cascade of effects including decreased inositol phospholipid metabolism, deranged PKC activity and Na\(^+\)-K\(^+\) ATPase activity (Figure 4). The initial depletion of MI has been associated with accumulation of sorbitol due to the activity of aldose reductase. The administration of aldose reductase inhibitors, including Sorbinil, correct the MI defect. However, the inositol catabolic pathway has been implicated in diabetic complications in lens tissue by Goode et al (Goode et al., 1996).

While the existence of MIOX has been known since the 1950’s, it has received little attention because it was thought to reside only in the kidney (Charalampous and Lyras, 1957; Koller and Hoffmann-Ostenhof, 1979; Bry and Hallman, 1991). Even a report of altered MIOX expression during diabetes (Whiting et al., 1979) has failed to
generate further research on the subject. Nearly all of the research on decreased MI has focused on correcting the sorbitol accumulation through aldose reductase inhibitors. In order to examine the possibility that MIOX plays a role in this process, it is necessary to determine if the enzyme is expressed solely in the kidney, or is found in other tissues; particularly those affected by diabetes.

Any examination of physiological roles for MI and MIOX, the prime enzyme in its catabolism, requires consideration of possible impact of MIOX-modulated MI concentrations on signal transduction pathways, which involve inositol phosphates. As seen from the review by Majerus (Majerus, 1992) the metabolism of inositol phosphates is quite complex and in mammalian systems is channeled through phosphatidylinositol as the common precursor of the important signaling molecules inositol 1,4,5-triphosphate and inositol 1,4-bisphosphate as well as diacylglycerol. Production of intracellular MI-derived second messengers is specifically involved in the control of cellular events, such as the spatial and temporal organization of key signaling pathways, the rearrangement of the actin cytoskeleton or intracellular vesicle trafficking (Payrastre et al., 2001). While little linkage has been drawn between MI concentrations and phosphoinositide metabolism, there is some evidence of altered signal transduction and inositol phosphate metabolism in rat sciatic nerves during streptozotocin-induced diabetes (Goraya et al., 1995). Since levels of MI in diabetic animals are reduced in the central nervous system, while renal clearance of MI is enhanced, kidney MIOX levels may play 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 MI
levels may be important in understanding diabetic pathologies at the level of phosphoinositide signaling.

While 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 (Palmano et al., 1977; Winegrad, 1987). Of the physiological isomers of inositol, MI and D-chiro-inositol are associated with diabetic pathologies (Winegrad, 1987; Larner et al., 1998). Intracellular MI depletion is associated with complications including diabetic nephropathy (Cohen et al., 1995; Cohen, 1986; Narayanan, 1993), retinopathy (Henry et al., 1993), neuropathy (Benfield, 1986; Dyck et al., 1989), and diabetic cataract (Goode et al., 1996; Lin et al., 1991). Most importantly, for the first time, the presence of MIOX in the mammalian lens has been demonstrated (Goode et al., 1996). It is the first direct evidence that MIOX may play a role in the secondary pathologies of diabetes, and given the apparent identity among the factors involved in neuropathy, nephropathy, and retinopathy (Bry and Hallman, 1991; Pfeifer and Schumer, 1995; Raccah et al., 1998), it is conceivable that MIOX may well be involved in all three pathologies. D-chiro-inositol is a component of an inositol phosphoglycan (IPG) that mediates the action of insulin (Fonteles et al., 1996). Infusion of the IPG containing D-chiro-inositol in streptozotocin diabetic rats corrected the hyperglycemic state (Fonteles et al., 2000; Oslund, Jr. et al., 1993). The discovery of a common transporter protein with specificity for both MI and D-chiro-inositol, which is inhibited by glucose, supports the increased clearance of both inositol isomers during hyperglycemia (Oslund, Jr. et al., 1996). Thus, MI and D-chiro-inositol appear to play a major role in the pathogenesis of
diabetes and its related complications of nephropathy, retinopathy, neuropathy and cataract.

While products of the glucuronate-xylulose pathway downstream from glucuronate have not been attributed with significant cellular signaling functions, glucuronate is integral to cellular functions such as glucuronidation of drugs and toxins (Gueraud and Paris, 1998), and as a component of the intracellular matrix. It is interesting that glucuronate produced by the catabolism of inositol by MIOX does not appear in these products (Hankes et al., 1969). Some mechanism is responsible for the immediate conversion of glucuronate to gulonate when inositol is the original substrate. Previously, the native MIOX enzyme was reported to be likely found in a complex with the enzyme responsible for the second step of MI catabolism, i.e. glucuronate reductase (Reddy et al., 1981a), which is also known as aldehyde reductase or ALR1 (EC 1.1.1.2) (De Jongh et al., 1987). It has been subsequently demonstrated that glucuronate reductase prefers the acyclic form of glucuronate (Naber and Hamilton, 1987). When glucuronate was replaced with stoichiometric equivalents of MIOX and MI reaction in the glucuronate reductase assays, a greater consumption of NADPH was observed than an equivalent amount of free glucuronate, which is predominantly in cyclic form. It is presumed that MIOX can transfer the acyclic glucuronate directly to the reductase in the complex (Reddy et al., 1981a). It was reasoned that ALR1 was trapping the glucuronate produced by MIOX before it could escape the complex to be utilized in other pathways.

In order to study the physiological relevance of the MIOX:ALR1 complex, first and foremost the expression pattern of MIOX must be established. Knowledge of the tissues and cell types in which MIOX is present will indicate whether the inositol
catabolism pathway fits the current diabetic complication pathogenesis models. Also, a model system is needed to explore how the MIOX expression is regulated \textit{in vivo}. Animal systems, especially streptozotocin-induced diabetic rats have been used widely as a model to study metabolic changes during diabetes. Just as widely used are cell culture models of specific tissues affected by diabetes. Understanding the localization of MIOX expression will allow for the selection and development of a useful model system for future studies. These experiments will form the basis for defining a suitable system for the study of MIOX.
Methods

Polyclonal Antibody Production

Recombinant MIOX protein was prepared as described previously (Chapter 1) to greater than 95% purity. Since the monoclonal antibodies prepared for the hog kidney purified MIOX had very low titers of monoclonal antibodies, purified recombinant MIOX was provided to Covance Inc. (Denver, PA) for the production of polyclonal antibodies in rabbit. After pre-immune control serum was collected, one rabbit was inoculated with 250µg recombinant MIOX. Booster inoculations of 125 µg were administered every 3 weeks. Test bleeds were taken twice of 7 ml serum. Production bleeds of 16 ml taken on days 75, 96, and 118. The sera were tested by Western blot analysis using recombinant MIOX for optimum concentration.

Tissue Homogenate Preparation

Hog tissues were obtained from a slaughterhouse within 10 minutes of death. Small pieces were snap frozen in liquid nitrogen and stored at −70 °C until use. Tissue protein preparations were made using mammalian protein extraction reagent (M-PER) as per manufacturer’s instructions (PIERCE). A 50 mg piece of each tissue was ground to paste using a plastic pestle in a 1.5 ml eppendorf tube. After addition of 1 ml M-PER reagent, the tissue was ground again and incubated at room temperature for 10 minutes. The tube was vortexed briefly and spun at 14,000 x g for 10 minutes to pellet debris. The supernatant was removed to a fresh tube and the protein concentration estimated by BCA Protein Assay (PIERCE) as per manufacturer’s instruction.
Rat and mouse kidneys were donated by other research groups and treated similarly as the hog tissues described above. Also, human kidneys were obtained from NDRI of Philadelphia, PA, by a specified protocol. Donor kidneys were obtained at the earliest possible time after death, normally 8-10 hours, and snap frozen in liquid nitrogen. Homogenate was prepared as described with the hog tissue.

**Western Immunoblot Analyses**

Western blot analysis to detect MIOX in tissue homogenates was done according to the procedure of Towbin et al. (1979). The protein was electrophoresed on an SDS-PAGE (12.5% acrylamide) and transferred to a nitrocellulose membrane (0.2 µm) at 100 volts for 90 minutes using Biorad minigel apparatus (Biorad, Hercules, CA). Following transblotting, the membrane was stained with Ponceau S to confirm uniform transfer of protein. The membrane was immersed in a blocking solution (TBS-T (0.05% Tween-20/0.1 M Tris/0.15 M NaCl, pH 7.5) containing 5% BSA). The membranes were hybridized with 1:15000 diluted MIOX polyclonal antisera in TBS-T containing 5% (w/v) BSA. The membranes were washed four times for 5 minutes with TBS-T, followed by hybridization to anti-rabbit IgG conjugated with horseradish peroxidase (1:2500) at room temperature for 60 min. The blot was washed three times in TBS-T and developed with the enhanced chemiluminescence detection method (Pierce).

Analysis of MIOX expression in the cell culture model was performed using the same method as described above. After transblotting, the membranes were stained with Ponceau S. The membranes were then cut into two pieces lengthwise at the 40 kDa marker protein band. The membrane fragment containing proteins less than 40 kDa was
analyzed for MIOX as described above. As a constitutively expressed control, the membrane containing proteins larger than 40 kDa was analyzed with polyclonal antisera for cyclooxygenase 1 (COX 1) at a dilution of 1:5000, then rehybridized with anti-rabbit IgG as done for MIOX. Each auto-radiograph was digitally imaged on an Eagle Eye II (Stratagene). Densitometry was performed with Scion Image software (Scion Corp.).

**In-Situ Hybridization Studies**

For localization and characterization of protein expression, tissue from cortex to outer medulla were dissected from kidneys and fixed in 4% paraformaldehyde. Sections of 0.5 µm thickness were prepared by Penn State EM Facility staff using a Leica Ultracut UCT microtome and mounted to slides. The slides were incubated with MIOX polyclonal antisera, and re-incubated with peroxidase-conjugated anti-rabbit IgG antibodies (sec) and DAB chromogen to visualize the MIOX protein. Digital images of slides were taken on a Nikon Eclipse TE300 microscope using Magnafire 2.0 software. Slides containing human tissue sections from a variety of organs were obtained from Novagen (Madison, WI). These slides were treated similarly to the kidney sections.

**Cell Model Studies**

The porcine kidney proximal tubule cell line LLC-PK1 was obtained from American Type Culture Collection (ATCC, Manassas, VA). Cells were cultured in M199 medium (Sigma Chemical Co, St Louis, MO) with 3% fetal bovine serum at 37 °C in a humidified atmosphere of 95% air and 5% carbon dioxide as per supplier’s
recommendations for 2 passages. The cells were split 1:5 at each passage, and half the cells were frozen at –70 °C in 10% DMSO to serve as a stock. New experiments were started from this frozen stock after one passage. For experiments to optimize MIOX expression, 50 µM MI was supplemented to the media as described by Kitamura et al. (Kitamura et al., 1998) for inositol transporter expression.

Cells were cultured on attachment substrates to optimize growth and MIOX expression. First, 6-well plates containing the attachment factors collagen I, collagen IV, fibronectin, laminin, and poly-D-lysine (BD Biosciences) were pre-warmed to 37 °C in an incubator. LLC-PK1 cells were cultured on the plates at 90% confluence. The media was changed every other day and the cells observed visually for growth and confluence. At one week and two weeks after confluence was reached, the cells were collected by scraping, washed twice in PBS buffer, and pelleted by centrifugation. Protein was extracted from the cell pellets using M-PER reagent as per manufacturer’s instructions and analyzed by western blot for MIOX expression.

The compounds TCDD, H2O2, dexamethasone, insulin, NaCl, PGJ2, and quercetin were tested for the ability to induce MIOX in LLC-PK1 cells. Two weeks before induction, 6-well plates pre-coated with collagen IV (BD Biosciences) were prewarmed to 37 °C. LLC-PK1 cells were cultured on the plates at 1x10^6 cells per well in 5 ml of M199 media (3% FBS, 50 µM MI). Media was changed every other day. Two weeks after plating, the cells were observed by microscope for confluence and dome (uplifted areas of cells due to fluid transport) formation. Each inducer was dissolved in appropriate solvent and sterilized by filtration through a 0.22 µm membrane. Then the induction compounds were mixed into 5 ml of culture media and added to cells with
gentle rocking. After 24 hours incubation, the media was aspirated, and the cells washed with PBS. The cells were scraped, pelleted by centrifugation, and protein extracted with M-PER. Western blot analysis was performed on the protein samples.

For hyperglycemia induction experiments, LLC-PK1 cells on 6-well plates were prepared similarly to the induction experiments described earlier. After 2 weeks of growth on collagen IV plates, the standard media was replaced on half the wells with the standard M199 media plus 25mM glucose. The remaining wells were maintained on standard media as control. Media was replaced every other day. Cells from one hyperglycemic and one control well were collected by scraping on days 1, 2, 4, 7, 10, and 14. Protein was extracted and Western blot analysis performed as before.
Results

Characterization of Polyclonal Antiserum

Polyclonal antibodies were raised against recombinant porcine MIOX expressed in BL21(DE3) bacteria. The expressed MIOX was purified to >95% homogeneity with only faint, low molecular weight bands visible on Coomassie stained SDS-PAGE gels. To characterize the antiserum, varying dilutions were hybridized to membranes containing 1 µg pure recombinant MIOX and 10 µg of hog kidney cytosol separated by SDS-PAGE. Binding capacity of the antiserum did not decline until dilutions greater than 1:15000 were used, indicating a high titer. Furthermore, dilutions of recombinant MIOX analyzed by western blot showed that the detection limit for porcine MIOX was 0.1 ng.

MIOX Expression in Kidneys

To determine whether the predominance of MIOX expression in the kidney is ubiquitous among the commonly used model species, the probes developed against porcine MIOX were used. Kidneys were obtained from hog, mouse, human, and rat sources and sections from cortex to medulla taken for study. The presence of both protein and mRNA for MIOX was then examined (Figure 13).

Western blots performed on kidney extracts taken primarily from cortex indicated that MIOX is present in most samples. When 8 µg of protein were loaded, bands corresponding to the recombinant MIOX were visible in all the species. For each species, other bands were detected. Particularly in hog, there are two bands of lower molecular
Figure 13. Analysis of kidney MIOX expression across species

A. Western blot of MIOX expression in kidneys. Lane 1, 1.5ng pure rMIOX; Lane 2, 8μg hog kidney cytosol; Lane 3, 8μg human kidney homogenate; Lane 4, 8μg rat kidney homogenate; Lane 5, 8μg mouse kidney homogenate. B. PCR amplification of MIOX mRNA from kidney tissues. Lane 1, 1kb PLUS marker; Lane 2, porcine kidney; Lane 3, mouse kidney; Lane 4, human kidney; Lane 5, rat kidney.
weight; one approximately 31 kDa and another about 29 kDa. These bands appear at similar intensity to the 32.7 kDa band. In human, the band estimated to be MIOX is of a slightly higher molecular weight (33.5 kDa), as calculated by the sequence published by Yang et al. (Yang et al., 2000). However, this band is relatively light compared to a major doublet band of about 31-32 kDa. In rat, there are two major bands; an intense 33.5 kDa band corresponding to the human MIOX, and an equally intense band of ~29 kDa. The band corresponding to mouse was very faint at the 8µg load. This lane also contained the most contaminating bands.

The presence of MIOX mRNA was also confirmed in kidneys of each species. PCR was performed with MIOX internal primers on both first strand cDNA as well as amplified cDNA. Mouse was the only species in which a strong band was detected on ethidium bromide stained agarose gel for MIOX from first strand cDNA. However, other species required the full-length cDNA to be PCR amplified using the SMART method (CLONTECH) before enough template was present to detect of MIOX transcript by subsequent PCR experiments.

*In situ* hybridization was employed to determine the spatial localization of MIOX in kidney. Sections of kidney cortex and outer medulla were hybridized with anti-MIOX serum. The staining (Fig 14a) shows that MIOX is located exclusively in tubular epithelial cells. No staining is present in the glomeruli (Fig 14a, arrows). In order to distinguish the types of tubular epithelium in which MIOX is present, antibodies against specific markers were used. Figure 14b, shows staining with antibodies to aquaporin 2, marker of collecting duct epithelia. Panel c shows Tamm-Horsfall protein (human uromucoid), a marker of distal tubule epithelia. MIOX does not co-localize with either of
these markers, indicating that MIOX is expressed exclusively in the proximal tubule epithelial cells, which make up the majority of cells in kidney cortex.

The \textit{in situ} hybridization was also performed on mouse and human sections. The signal was relatively weak in both tissue types (1:2000 dilution antiserum). This corresponds to the relatively weak signal obtained for MIOX on western blots. In mouse (Fig. 15a), the staining is similar to hog, with the tubular epithelial cells being the exclusive location of staining. The glomeruli show no presence of MIOX. Human kidney sections also stain for MIOX with the same pattern as seen in the case of hog (Fig 16a), however the pattern is more diffuse. The glomeruli do not stain, nor to the larger collecting duct tubules. The proximal tubules are less defined than in hog and mouse, but this is where the staining is localized.
Figure 14. Localization of MIOX in hog kidney

*In situ* hybridization was performed on hog kidney sections hybridized to antibody against the indicated protein. Positive staining is indicated by dark red areas. Arrows in the MIOX section indicate glomeruli.
Figure 15. *In situ* staining of MIOX in mouse kidney

A. Normal mouse kidney section hybridized with anti-MIOX serum.  B. Diabetic mouse kidney
Figure 16. *In situ* staining of MIOX in human kidney

MIOX Screening of Tissues

The distribution of MIOX in non-kidney hog tissues was determined by both PCR and Western blot analysis. Western blots indicate that MIOX is not expressed in any of the tissues except for low levels in retina (Fig. 17a). Northern blot indicates abundant mRNA in hog kidney but not in other tissues (Fig. 17b). RT-PCR also failed to detect MIOX transcript in any of the non-kidney samples (data not shown). However, retina and lens could not be examined by Northern blot or PCR due to the method of collection. In order to obtain tissue for westerns, the outer layers of the hog eye had to be thawed quickly in warm water, followed by partial removal of the cornea followed by removal of the frozen vitreous humor. This exposed the retina for sampling. However, the relatively harsh treatment precluded the possibility of preparing samples for RNA extraction.

Cell lines were examined to fill in missing information from the hog tissue experiments. A novel human lens epithelial cell line, HLE-B3, was kindly donated by Dr. Usha Andley from Washington University School of Medicine. This cell line demonstrated MIOX protein by western blot (Figure 17c), as well as message by RT-PCR. Total RNA was obtained from a human retinal pigmented epithelial cell line cultured in Dr. Sylvia Smith’s lab at the Medical College of Georgia. RT-PCR indicated a light positive for MIOX message, and western blot analysis to be performed in the future. Finally, the expression pattern of MIOX in human tissues was examined by in situ hybridization on commercially available slides (Novagen). The only significant staining is present on the kidney section (Fig. 18).
Figure 17. **Western blot analysis of tissue distribution of MIOX**

A. Western immunoblot of hog tissue screening for MIOX. Lane 1, Markers; Lane 2, blank; Lane 3, liver; Lane 4, heart; Lane 5, lung; Lane 6, lens; Lane 7, retina; Lane 8, rat kidney; Lane 9, mouse kidney. The load per lane was 20µg protein. White circle indicates positive for retina. B. Northern blot of hog tissue mRNA using MIOX specific probe. Lane 1, kidney; Lane 2, lung; Lane 3, liver; Lane 4, ovary. mRNA load per lane was 20 µg. C. Western blot of the human lens epithelial cell line HLE-B3. Lane 1, HLE-B3; Lane 2, Human kidney. Arrow indicates ~33 kDa MW. Protein load: 20µg.
Figure 18. Human tissue screening for MIOX by *in situ* hybridization

Human tissue sections (Novagen) were hybridized with anti-MIOX serum.
LLC-PK1 Cell Model

The proximal tubule cell line LLC-PK1 was grown as per supplier’s indications for 2 passages. Attachment factors were tested for their effects on the growth and differentiation of cells as well as MIOX expression. Cells cultured on an uncoated plastic surface do not halt growth upon forming a confluent monolayer. Some growth continues, as evidenced by the appearance of stacked areas of cells that eventually detach from the plate. Growth on attachment factors including collagen IV, collagen I, laminin, fibronectin, and poly-D-lysine was faster than cells plated on plastic culture dishes. Also, once the cells reached confluence, growth stopped. After confluence, domes of cells were observed, indicating that the cells had fully differentiated and were transporting fluid unidirectionally to below the monolayer. Summaries of the observations are on Table 6. Western Blot analysis indicated that the attachment factors caused relatively little change in MIOX expression (Fig. 19). After 2 weeks, culture on collagen IV improved the expression of MIOX over uncoated control by 1.13 fold (N=2). The fibronectin-plated cells began to detach and die shortly thereafter. Confluent monolayers on collagen IV were observed and found stable out to 50 days.
Table 6. Observational summary of attachment factor effects on LLC-PK1

<table>
<thead>
<tr>
<th>Attachment Factor</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uncoated</td>
<td>Growth beyond confluence</td>
</tr>
<tr>
<td>Collagen I</td>
<td>Growth, differentiation after confluence; dome formation Poor survival after 2 weeks</td>
</tr>
<tr>
<td>Collagen IV</td>
<td>Growth, differentiation after confluence; dome formation Stabile past 50 days</td>
</tr>
<tr>
<td>Laminin</td>
<td>Growth, differentiation after confluence; dome formation Adherence degrades after 1.5 weeks</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>Growth, differentiation after confluence; dome formation Stabile for 50 days</td>
</tr>
<tr>
<td>Poly-D-Lysine</td>
<td>Growth, differentiation after confluence; no dome formation death after 2 weeks</td>
</tr>
</tbody>
</table>
Figure 19. The effect of attachment factors on MIOX expression in LLC-PK1 cells

Western Blot analysis of the effect of attachment factors on the expression of MIOX in LLC-PK1 cells. Lane 1, no attachment factor; Lane 2, collagen I; Lane 3, collagen IV; Lane 4, laminin; Lane 5, fibronectin; Lane 6, poly-D-lysine. Protein load was 25µg per lane.
The culture conditions and collagen IV coated plates were used to examine the effects of some compounds on the induction of MIOX in LLC-PK1 cells. After 2 weeks growth on collagen IV, the cells were treated for 24 hours with various inducers (Table 7), and analyzed by western blot. Insulin treatment at 25µg/ml caused a slight increase in MIOX expression (~2 fold). No other compounds caused a significant change. A range of insulin from 250ng/ml to 125µg/ml was applied to cells to determine optimum dose for increased MIOX expression, as seen in Figure 20. All doses caused a similar ~2 fold increase in MIOX expression levels.

Finally, the effects of hyperglycemia on MIOX expression in LLC-PK1 cells were examined. After two weeks culture on collagen IV plates, the media was changed to hyperglycemic similar to diabetic plasma levels (25mM). After 1 and 2 weeks in hyperglycemic media, cells demonstrated hypertrophy, as reported by (Yang et al., 1997). Western blot analysis of the protein levels indicated no significant differences in MIOX expression. The hyperglycemic samples fluctuated, but in no reproducible manner. These experiments could not be continued beyond 2 weeks, as the cells began to detach from the substrate and die.

**Mouse and Human Diabetes**

Tissue models of diabetes were examined for MIOX levels. Mouse kidneys were obtained as a gift from Dr. Douglas Cavener that were wild type and a knockout strain for the enzyme eIF2α kinase (PERK). Mice that are PERK -/- lose function of pancreatic islet cells postnatally (Harding et al., 2001). By 3-4 weeks of age they become acutely
Figure 20. The effect of insulin on MIOX expression in LLC-PK1 cells

Western Blot analysis of the effect of insulin treatment on MIOX expression in LLC-PK1 cells. Lane 1, control; Lane 2, 250 ng/ml insulin; Lane 3, 5 µg/ml insulin; Lane 4, 25 µg/ml insulin; Lane 5, 50 µg/ml insulin; Lane 3, 125 µg/ml insulin. Protein load was 25 µg per lane.
Table 7. Effects of compounds on MIOX expression in LLC-PK1 cells.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Effect on cell growth</th>
<th>MIOX expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 nM TCDD</td>
<td>No different from control</td>
<td>Same as control</td>
</tr>
<tr>
<td>50 µM H₂O₂</td>
<td>Some cell death</td>
<td>Same as control</td>
</tr>
<tr>
<td>10 µM dexamethasone</td>
<td>No different from control</td>
<td>Same as control</td>
</tr>
<tr>
<td>25 µg/ml insulin</td>
<td>No different from control</td>
<td>2.1 fold induction</td>
</tr>
<tr>
<td>300 mM NaCl</td>
<td>Massive cell death</td>
<td>N/D</td>
</tr>
</tbody>
</table>
hyperglycemic. Six samples were obtained (Table 8), three each of PERK +/+ and PERK -/- for western blot analysis and in situ hybridization. Average MIOX protein levels were higher in diabetic animals, but not statistically significant ($p>0.6$). In situ results also demonstrated a wider expression pattern of MIOX in the diabetic kidney sections (Fig. 15b).

Human kidneys were obtained from cadavers of patients that had died from myocardial infarction. All of the donors were aged >50, and disease of diabetic subjects was of unknown duration. In situ hybridization of one diabetic patient suffering from renal failure (Fig. 16b) indicates that the kidney cortex has suffered massive cell death and that the proximal tubules are indistinguishable, and there is little detectable MIOX expression.
Table 8. Glucose status of PERK knockout mice

<table>
<thead>
<tr>
<th>Mouse</th>
<th>PERK Genotype</th>
<th>Glucose (mg/dl)</th>
<th>Age (days)</th>
<th>Relative MIOX signal (MIOX:COX 1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3213</td>
<td>-/-</td>
<td>&gt;600</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td>3214</td>
<td>-/-</td>
<td>&gt;600</td>
<td>34</td>
<td>0.82</td>
</tr>
<tr>
<td>3252</td>
<td>-/-</td>
<td>596</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>3215</td>
<td>+/-</td>
<td>108</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td>3282</td>
<td>+/-</td>
<td>104</td>
<td>25</td>
<td>0.70</td>
</tr>
<tr>
<td>3284</td>
<td>+/-</td>
<td>59</td>
<td>25</td>
<td></td>
</tr>
</tbody>
</table>
Discussion

MIOX has been reported to be expressed exclusively in the mammalian kidney in the few species it has been identified in to date (Charalampous, 1959; Koller and Koller, 1990; Reddy et al., 1981a). The early searches focused on finding rich sources of enzyme activity for purification and study. Interest in the enzyme was generated by its unique activity and mechanism. The problems with detecting activity in crude tissue homogenates due to interference by sugars with the orcinol assay likely would have prevented MIOX from being discovered in other mammalian sources. Indeed, the only other reported non-kidney source has been in oat seedlings (Koller et al., 1976).

First, I sought to confirm the existence of MIOX in kidneys from several species. The only characterized sources of MIOX activity has been in rat (Charalampous, 1959; Koller and Hoffmann-Ostenhof, 1979; Koller and Koller, 1984) and hog (Reddy et al., 1981a; Reddy et al., 1981b). Using MIOX specific polyclonal antibody and PCR primers specific for porcine MIOX as probes, the distribution of MIOX was examined not only rat and hog, but also mouse and human tissues. The results indicate the existence of MIOX protein and mRNA in all the species, suggesting that MIOX is likely found ubiquitously in kidney of all mammalian species. However, while all kidneys examined appear to be relatively rich in MIOX protein, the specificity of the polyclonal antibodies raised against porcine MIOX varies somewhat in cross-reactivity with MIOX from other species. While it reacts strongly with rat MIOX and less so with the human protein, its reactivity to mouse MIOX is relatively weak. Species differences in sequence are likely involved.
The localization of MIOX in kidney was examined next. *In situ* hybridization showed a complete lack of MIOX protein in the glomeruli of kidney sections (Figure 14). The cortex tubules, however, were rich with MIOX. There are three types of tubule epithelial cells present in cortex; the proximal convoluted tubule, distal tubule, and cortical collecting duct. Specific marker proteins were used to distinguish the distal and collecting duct cells. The staining pattern indicated that MIOX is expressed exclusively in the proximal convoluted tubule, which is in agreement with the results published for a MIOX-like protein in mouse (Yang et al., 2000). Proximal tubules are highly active metabolically. These cells are responsible for reabsorption of 70% of the water and salt from the glomerular filtrate, as well as nearly 100% of metabolites such as glucose, other sugars and MI. There are some possibilities for the role of MIOX in epithelial cells. First, the main role may be to catabolize excess MI in plasma. MI is synthesized by many cell types in sufficient quantities to maintain homeostasis, and diet increases the levels. It has been estimated that MIOX activity in kidney is equivalent to the daily dietary intake of MI (Weinhold and Anderson, 1967). Another function may be in the regulation of cellular signaling through inositol phosphates by regulating the MI pool. Tubular cells must grow and divide to fill gaps in the tubules left by damage and dead cells. MIOX may regulate growth and differentiation by catabolizing MI, thus altering the availability of inositol for inositol phosphate biosynthesis.

If MIOX is involved in regulation of cell signaling, it is likely that MIOX is expressed in other tissues and cell types beyond just kidney. The pathways of phosphoinositide metabolism are ubiquitous in all tissues, so the possibility of other cell types similar to those found in kidney may express MIOX as a mechanism to regulate
intracellular MI pools. Both western analysis and in situ hybridization failed to detect MIOX in any other major organ. However, MIOX was detected in retina, as well as lens epithelial cells. Goode (Goode et al., 1996) has previously reported MIOX activity in rat lens. These findings are significant as both of these tissues, along with kidney, are subject to complications of diabetes. Altered MI metabolism is involved in the pathogenesis of diabetic complications in these tissues. These findings leave open the possibility that altered expression of MIOX may play a role in the cellular damage arising from NIDDM. However, due to our results with the cell line LLC-PK1 and the modification of MIOX by PKC, the possibility that MIOX expression may remain constitutive. Post-translational modifications and localization of the MIOX:ALR1 complex may determine the effect MIOX has on intracellular MI pools.

We attempted to develop a cell culture model in order to study how MIOX is expressed. The hog proximal tubule cell line LLC-PK1 was chosen because it expressed detectable levels of MIOX protein. While some improvement in the expression of MIOX was found when the cells were cultured on collagen IV, the expression was still very low. None of the compounds tested except for insulin was able to alter the expression levels. Culturing cells in hyperglycemic media also failed to elicit any significant changes in MIOX levels. Taken together, these results indicate that LLC-PK1 cells may not serve as a good model for studies of MIOX expression. Another group has also reported that cell lines do not express MIOX-like protein (Hu et al., 2000). Even cultured kidney cells quickly lost the ability to express MIOX. However, there is some evidence that MIOX expression is changed in vivo. Diabetes was reported by Yang et al (Yang et al., 2000) to
increase MIOX-like message levels significantly in mice. Studies into the physiological role of MIOX may have to be conducted in whole animal models.

A preliminary examination of two such models was undertaken in mice and human samples. PERK knockout mice failed to demonstrate any significant difference in MIOX expression levels. The experiment was complicated by two factors: the low cross reactivity of the polyclonal antisera and the age of the mice. Increased loading of the SDS-PAGE minimized the first problem. The second problem lies in the development of diabetes in PERK knockout mice. Even though the mice were acutely hyperglycemic at the time of sampling, it is unknown how long they had been diabetic. The subject group therefore represented a “snapshot” of the disease, without context to the progression of the disease. Similar problems presented themselves with the human samples as well. The duration of the diabetes of the patients could not be determined. All of the diabetic kidneys had some necrosis, and it is unknown, except for one individual, what was the status of their kidney function.

In conclusion, MIOX may play a role in the pathogenesis of diabetic complications. The enzyme is expressed in cells outside kidney, in epithelial cells of the lens and retina. All of these cell types are affected by diabetic complications. Unfortunately, cell culture models do not seem suitable for the study of MIOX expression. The best system would be a whole animal model such as hog or rat in which diabetes can be induced and MIOX expression followed through the progression of disease.
Summary and Conclusions

The cloning and characterization of MIOX presented in this thesis research is the first report and these results provide confirmation of many of the properties of the purified native MIOX reported in previous studies, and also clarifies some of the discrepancies among those reports. Recombinant porcine MIOX purified from bacterial expression systems have shown similar kinetic and activation properties as the native enzyme. The \( K_m \) value for MI substrate was nearly identical at 5.9mM (5mM reported for native porcine MIOX (Reddy et al., 1981a)). Also significant, but expected, was the requirement of ferrous iron and cysteine by the purified enzyme. Non-heme iron is responsible for the activation of molecular oxygen in the active site of many oxygenases (Bugg, 2001). In fact, many iron requiring oxygenases, such as methane monooxygenase (Kopp et al., 2001), lipoxygenases (Nelson and Seitz, 1994), and chlorocatechol dioxygenase (Broderick et al., 1993), have been characterized extensively. Purification eliminated all detectable enzyme activity in the expressed MIOX protein, presumably through loss of iron from the active site.

Perhaps more important than the established properties of MIOX, are the properties for which there is considerable conflicting evidence in the literature. The most obvious is the molecular mass of MIOX. As seen in Table 1, the reported molecular mass for rat MIOX has been identified as 68 kDa (Charalampous, 1959) and \(~17\) kDa (Koller and Hoffmann-Ostenhof, 1979) by separate laboratories. Reddy et al (Reddy et al., 1981a) identified porcine MIOX as a 66 kDa protein. In fact, the true molecular mass
is established conclusively for porcine MIOX as 32.7 kDa by our studies. Interestingly, none of the other studies agree with our result, however, all of the reported values are either approximately 2 fold the true molecular mass or one half the mass. This would suggest that MIOX may dimerize or oligomerize, but analytical centrifugation failed to detect this in the recombinant protein, even in the presence of Fe/Cys and substrate. It should also be noted that similar genes in human, rat and mouse identify MIOX as a 33 kDa product. This was confirmed in mouse by bacterial expression, as the mouse rMIOX exhibited activity towards MI.

MIOX is a very interesting enzyme in its unique mechanistic aspects, as it is a monooxygenase that catalyzes a glycol cleavage. However, its physiological relevance is of equal or greater interest due to the importance of inositol metabolism and its potential impact on disease. First and foremost, MI has been established as an essential factor in growth and development of embryos (Akashi et al., 1991; Hod et al., 1986), and in the development of specific organs (Hallman et al., 1986; Bry and Hallman, 1991). Depletion of MI in embryos has been identified with developmental defects (Akashi et al., 1991) and in diabetic embryopathy (Hod et al., 1986). One of MI’s most crucial roles is in cell signaling pathways, through its incorporation into phosphatidylinositol and further phosphorylation to yield other intermediates of signal transduction (PIP, PIP₂, etc) (Downes and Macphee, 1990; Loewus and Loewus, 1983; Winegrad, 1987). Cascades resulting from the release of these phosphoinositides by protein kinase C affect a large spectrum of signaling pathways. Some of these pathways likely involve lipid metabolism, as MI has been characterized as a lipotropic factor (Shepherd and Taylor, 1974; Khatami, 1988). MI supplementation has been shown to affect the fatty liver
condition (Katayama, 1999). Finally, the pathway of catabolism of inositol supplies substrate for the synthesis of ascorbic acid (Allison and Stewart, 1973) in animals such as mouse and rat, but not in humans, which lack the necessary enzyme.

Taking into account the diverse and essential roles of inositol in the pathways described above, it is reasonable to expect that catabolism of inositol may affect those pathways. In this report I have identified tissues other than kidney that show some expression of MIOX, particularly the retina and human lens epithelia. I have also confirmed the localization of MIOX in porcine kidney to the proximal tubular epithelial cells. While the cellular localization in porcine retina was not determined, it is likely that the retinal pigmented epithelial cells are the site of MIOX activity, since both ALR1 expression (Poulsom, 1986) and diabetes induced MI depletion (Thomas et al., 1993;Benfield, 1986) have been identified in these cells. Other localization studies of ALR1 indicate (Terubayashi et al., 1989;Akagi et al., 1987) that the reductase is present in the same tissue as MIOX. These findings suggest the possibility that the MIOX-ALR1 complex is specific to epithelial cells and may be found in other epithelial cells as well. If so, the function of the complex is likely to regulate the levels of inositol available for PI synthesis and growth regulation. It has been observed that MIOX becomes active after birth in rabbit (Bry and Hallman, 1991), with a steady increase in activity to adulthood. As growth requirements decrease, MIOX activity likely reduces the inositol pools in epithelial cells available for PI synthesis, resulting in a down regulation of growth responses. With ALR1 closely associated with MIOX, the glucuronate produced by inositol catabolism is shunted down the glucuronate-xylulose pathway to enter the pentose phosphate pathway and energy metabolism, instead of being released to serve as
substrate for the production of structural aminoglycans needed in growth processes. The observations I report on a small increase in MIOX protein after LLC-PK1 cells reach confluence and maturation support this notion.

All of the tissues that MIOX has been observed in undergo metabolic derangements during diabetes. The presence of MIOX in three of the major tissues that experience complications of NIDDM is a strong indicator that MIOX may potentially play a role in the MI depletion associated with pathogenesis of these complications, and future studies will examine the peripheral nerve cells for MIOX expression. The mechanism behind the depletion of MI in tissues during NIDDM has remained a mystery (Winegrad, 1987) for over 20 years. Since the MI depletion almost always accompanies increased polyol pathway activity, the focus has remained on the role of aldose reductase. Numerous aldose reductase inhibitors (ARI) have been synthesized, with varying effects on the pathogenesis of diabetic complications. Little study has been performed on the inhibitory affect of these compounds on the activity of ALR1, a closely related enzyme to aldose reductase. The most studied aldose reductase inhibitor, Sorbinil, is also an inhibitor of ALR1 activity, although not as potent. My results indicate that Sorbinil can inhibit ALR1 found in complex with MIOX from hog kidney. While not all activity towards glucuronate is abolished, the activity of the complex with MI as substrate is decreased to undetectable levels. The slow turnover rate of MIOX means that a limited amount of acyclic glucuronate becomes available to ALR1, and significant inhibition of ALR1 probably prevents the enzyme from utilizing the substrate before it either forms the hemiacetal or is released from the complex to the cytosol (Fig. 21).
Figure 21. The acyclic and hemiacetal forms of D-glucuronate.
Since Sorbinil does not inhibit MIOX directly, treatment of diabetic tissues with ARIs should not affect the catabolism of MI. If MIOX is implicated in the depletion of MI in these tissues, the correction of MI metabolism by ARIs must be explained. The answer may come from the seldom studied metabolite glucarate. Glucarate is produced from glucuronolactone either enzymatically in the liver (Tonkes and Marsh, 1973), or nonenzymatically by a free radical mechanism (Marsh, 1986), and is the only naturally occurring carbohydrate identified to inhibit MIOX. High endogenous glucarate has been reported to be a consequence of the loss of ALR1 activity, either from a metabolic defect (Westall et al., 1970), or due to inhibition of ALR1 by ARIs (Hoyle et al., 1992). In either case, the loss of ALR1 activity was followed by increased excretion of both glucuronate and glucaric acid in the urine, although the affect was not immediate in rats given ARIs. Hinohara et al (Hinohara et al., 1974) has reported that the transformation of glucuronolactone to glucarate is more efficient in alloxan induced diabetic rabbits. $^{14}$C-labeled glucuronolactone was converted to glucarate much faster compared to normal controls. It could be speculated that when ARIs are administered to treat diabetic complications, the ALR1 that is in complex with MIOX is inhibited. Glucuronate then produced by MIOX escapes in the cytoplasm, where it is converted to glucuronolactone and glucarate. The oxidative stress caused by diabetes leads to conversion of free glucuronate to glucarate through a free radical mechanism (Marsh, 1986). Increasing levels of glucarate then inhibit MIOX, leading to the normalization of the MI depletion defect observed. In this model the true role of ARIs in the treatment of diabetic complications is found in its inhibition of ALR1, not the correction of polyol pathway
activity through ALR2. Side effects found in the administration of ARIs to humans in the
treatment of diabetes may be avoided if MIOX activity is inhibited directly.

While the focus of these conclusions has been on the possible relevance of MIOX
to diabetic complications, this is not the only area of potential importance. It has been
observed in some studies that MI supplementation decreases lung tumorigenesis (Hecht
et al., 1999; Hecht et al., 2001). Phytic acid, an inositol with all carbons phosphorylated,
has also been identified to possess anticarcinogenic properties (Morrison et al.,
2002; Midorikawa et al., 2001). It may be of value to examine tumor tissues to MIOX
expression and evaluate the role that the inositol catabolic pathway may play.

In conclusion, the results reported here form a foundation for not only the detailed
mechanistic characterization of MIOX, but also the study of the physiological role of
MIOX. The successful cloning and expression of MIOX allows sufficient amounts of
pure protein to be produced for characterization of the mechanism and crystal structure.
The characterization of the recombinant MIOX confirms the identity of this protein with
the previously reported porcine MIOX (Reddy et al., 1981a). It also elucidates the true
molecular mass and oligomerization properties, which have been contradictory in
previous studies. The localization of MIOX has also been elucidated. MIOX is
expressed predominantly in the kidney, but is also present in retina, and lens epithelial
cells. All of these tissues are susceptible to the complications of Diabetes Mellitus.
Unfortunately, kidney epithelial cell lines were found to be unsuitable as models for the
study of MIOX expression. While the cells do express a low amount of MIOX, the levels
are much less than found in tissue preparations. Also, MIOX in LLC-PK1 cells was
found to be unresponsive to any treatments tested. These results are in agreement with a
previous study (Hu et al., 2000) which found not only did cell lines did not express MIOX-like protein, but cultured cells quickly lose the ability to express MIOX. Examination of human and mouse diabetic kidneys indicates that a proper in vivo model must be examined from induction of diabetes to the pathogenesis of complications. The most promising models are streptozotocin induced diabetic rodents or swine.
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