L-*myo*-Inosose-1 as a Probable Intermediate in the Reaction Catalyzed by *myo*-Inositol Oxygenase†

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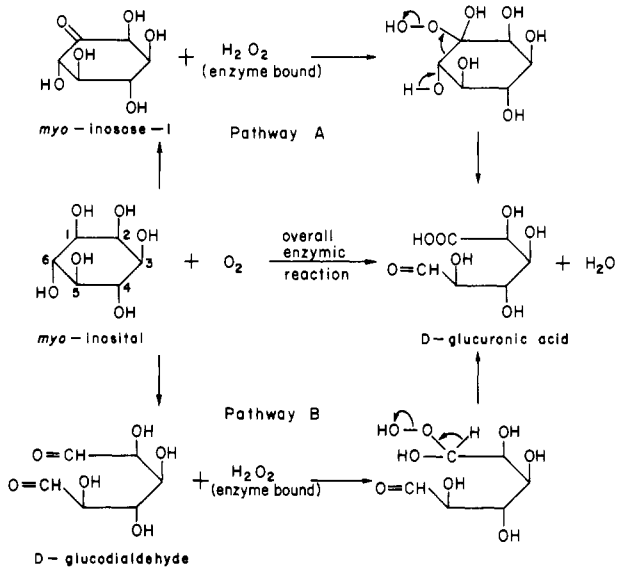
ABSTRACT: In previous investigations, it was necessary to have Fe(II) and cysteine present in order to assay the catalytic activity of purified hog kidney *myo*-inositol oxygenase. In the present study it was found that, if this purified nonheme iron enzyme is slowly frozen in solution with glutathione and stored at −20 °C, it is fully active in the absence of activators if catalase is present to remove adventitious H_2O_2. With this simpler assay system it was possible to clarify the effects of several variables on the enzymic reaction. Thus, the maximum velocity is pH-dependent with a maximum around pH 9.5, but the apparent K_m for *myo*-inositol (air atmosphere) remains constant at 5.0 mM throughout a broad pH range. The enzyme is quite specific for its substrate *myo*-inositol, is very sensitive to oxidants and reductants, but is not affected by a variety of complexing agents, nucleotides, sulfhydryl reagents, etc. In other experiments it was found that L-*myo*-inosose-1, a potential intermediate in the enzymic reaction, is a potent competitive inhibitor (K_i = 62 μM), while other inososes and a solution thought to contain D-glucodialdehyde, another potential intermediate, are weak inhibitors. Also, both a kinetic deuterium isotope effect (k_H/k_D = 2.1) and a tritium isotope effect (k_H/k_T = 7.5) are observed for the enzymic reaction when [1-^{2}H]- and [1-^{3}H]-*myo*-inositol are used as reactants. These latter results are considered strong evidence that the oxygenase reaction proceeds by a pathway involving L-*myo*-inosose-1 as an intermediate rather than by an alternative pathway that would have D-glucodialdehyde as the intermediate. The other evidence suggests that the catalytically active form of the enzyme is a reduced species that can be reversibly converted to a catalytically inactive oxidized form but that higher valence states of the iron are probably involved during catalytic turnover, especially in the alcohol to ketone step.

Hog kidney *myo*-inositol oxygenase (EC 1.13.99.1), an enzyme that catalyzes the reaction of *myo*-inositol with O_2 to give D-glucuronate, has recently been purified to homogeneity in our laboratories (Reddy et al., 1981b), and some of its characteristics have been determined (Reddy et al., 1981a; Reddy & Hamilton 1981; Hamilton et al., 1982). This enzyme and its reaction are of considerable biological interest for several reasons, including (a) in animals, the enzyme is present exclusively in the kidney and it catalyzes the first committed step in the only known pathway of inositol catabolism (Charalampous & Lyras, 1957; Charalampous, 1959; Howard & Anderson, 1967), (b) the enzyme activity is known to be decreased in diabetic animals (Whiting et al., 1979), (c) recent indications that various inositol derivatives act as second messengers in mammalian cells (Michell et al., 1981; Berridge & Irvine, 1984; Hokin, 1985; Parthasarathy & Eisenberg, 1986) suggest that the inositol status of animals, partly controlled by this enzyme, is of special importance, and (d) a related enzyme plays a major role in plant metabolism (Loewus & Loewus, 1983).

myo-Inositol oxygenase is also of interest mechanistically because currently its mechanism is unknown and the reaction the enzyme catalyzes is unique; no other enzyme of which the authors are aware catalyzes a similar reaction. For these reasons we have recently been investigating its mechanism. It has been determined from tracer experiments (Moskala et al., 1981) that the enzyme is a monooxygenase; only one atom from O_2 is incorporated into the D-glucuronic acid, and it is specifically incorporated into the carboxyl group. Furthermore, the enzyme contains nonheme iron as its only known cofactor (Charalampous, 1959; Koller & Hoffman-Ostenhof, 1979; Reddy et al., 1981b).

Scheme I



Since an enzymic reaction as complicated as the one catalyzed by inositol oxygenase must proceed with several enzyme-bound intermediates, we have recently been attempting to characterize what these might be. On the basis of reasonable chemical analogies (Hamilton, 1969, 1971, 1974; Fraser & Hamilton, 1982) the two most likely pathways that

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are consistent with the $^{18}O_2$ tracer results would seem to be those illustrated as A and B in Scheme I. Since the overall reaction involves oxidation at the 1-position as well as oxidative carbon–carbon bond cleavage, these two 2-electron steps could occur in different sequences, i.e., as in pathway A, where the carbon–carbon bond cleavage occurs after initial oxidation at the 1-position to L-*myo*-inosose-1, or as in pathway B, where the cleavage occurs to give D-glucodialdehyde prior to oxidation at the 1-position. In either case, the O_2 would be reduced in the first step to the peroxide oxidation level, but whether it is enzyme bound H_2O_2 itself, as written in the scheme, or some other species is currently unknown. In the present research we have attempted to distinguish between alternatives A and B by studying the effects of the proposed intermediates on the rate of the enzyme catalyzed reaction and by investigating the kinetic isotope effects observed when the reactant *myo*-inositol is labeled with either ^2H or ^3H at the 1-position. The results strongly imply that the reaction is proceeding by pathway A.

In previous investigations with the purified oxygenase, it was necessary to assay for catalytic activity in the presence of activators such as Fe(II) and cysteine; in their absence the purified enzyme was virtually catalytically inactive. During the course of the present work, methods were developed to obtain enzyme that is fully active in the absence of activators. Some catalytic properties of the purified enzyme have been reexamined by using this simpler assay system, and the results of these studies are also reported.

Experimental Procedures

Unless otherwise specified, these were the same as previously described (Reddy et al., 1981a,b). A more complete description is given by Swan (1982) and Naber (1983).

Materials. The virtually homogeneous hog kidney inositol oxygenase was obtained by a modification of the earlier purification procedure (Reddy et al., 1981b) in which the gel filtration step was carried out on a Sephacryl S-200 column rather than the Bio-Gel P-150 column. Enzyme prepared in this way was greater than 80% pure, as judged by sodium dodecyl sulfate and analytical gel electrophoresis, and had catalytic activity comparable to that previously reported. Following purification, the enzyme (ca. 0.5 mg/mL) in 50 mM acetate buffer, pH 6.0, containing 50 mM KCl and 1 mM glutathione was divided into 0.3-mL portions and stored at −20 °C.

L-Inositol, obtained as a gift from Dr. L. Anderson, University of Wisconsin, was used to prepare L-*myo*-inosose-1 and its phenylhydrazone by procedures given by Post and Anderson (1962). *myo*-Inosose-2 and DL-*epi*-inosose-2 were prepared as described by Posternak (1952). A solution thought to contain D-glucodialdehyde was prepared by allowing 0.56 mmol of L-inositol to react with 0.28 mmol of $NaIO_4$ in 5 mL of H_2O for 24 h at room temperature.

[1-^2H]- and [1-^3H]-myo-Inositols. These were prepared by reduction of solutions of L-*myo*-inosose-1 (prepared from its phenylhydrazone), using labeled sodium borohydride. The procedure gives an ca. 50:50 mixture of *myo*-inositol and L-inositol that was separated by chromatography on cellulose.

To a suspension of 1.2 g of L-*myo*-inosose-1 phenylhydrazone in 10 mL of ethanol containing 1 mL of benzaldehyde and 0.4 mL of glacial acetic acid was added 25 mL of boiling water, and the mixture was heated in a boiling water bath for 10 min. After the mixture was cooled to 4 °C, the benzaldehyde phenylhydrazone was removed by filtration, the filtrate extracted twice with 30-mL portions of ether, and the aqueous layer concentrated under reduced pressure to ca. 10 mL. When analyzed by TLC[1] on cellulose plates with elution by an 80:20 acetone–water mixture (visualization by spraying with 0.1 N $AgNO_3$/5 N NH_4OH/2 N NaOH, 1:1:2), this solution showed only one spot with the same R_f as authentic crystalline L-*myo*-inosose-1. (The L-*myo*-inosose-1 was usually not obtained in crystalline form because it is difficult to crystallize in high yield.)

For the preparation of [1-^2H]-*myo*-inositol, 0.2 g of sodium borodeuteride (98% D; Alfa) was added to the above 10 mL of L-*myo*-inosose-1 solution; the solution was stirred for 24 h at room temperature and then acidified with 1 N HCl to pH 2–3. The resulting mixture was placed on a column (2.5 × 50 cm) packed with 20 g of each of the following Amberlite ion exchangers (Sigma Chemical Co.) in order from top to bottom: IRA-743 (to remove borate ion), IRA-45, and IRA-118H. (Prior to packing the column, the ion-exchange resins were prepared for use as follows: IRA-118H by soaking with sufficient 1 N HCl to maintain a low pH in the supernatant fluid and then washing with distilled water until the pH of the wash water was neutral; IRA-743 and IRA-45 by soaking with sufficient 1 N NaOH to give a basic pH and then washing with distilled water until the wash water was neutral.) After the mixture was eluted with 200 mL of H_2O, the eluent was concentrated under reduced pressure to ca. 4 mL and placed on a cellulose column (1 × 50 cm) prepared from 25 g of microgranular CC_{41} cellulose powder thoroughly washed with an 80:20 acetone–water mixture. This same solvent was also used to elute the column; 3-mL fractions were collected and analyzed by TLC for *myo*- and L-inositol, using analytical cellulose plates eluted with the 80:20 acetone-water mixture, and visualized as described above for *myo*-inosose-1. Fractions 12–39 contained L-inositol as the only component, and fractions 63–90 contained only *myo*-inositol as indicated by comparison of their R_f's with those of authentic samples. Fractions 40–62, which contained a mixture of the two inositols, were combined, concentrated under reduced pressure to 4 mL, and applied to the same column, and the procedure was repeated. Fractions from the two elutions that contained only pure [1-^2H]-*myo*-inositol were combined, evaporated to dryness, washed with 10–15 mL of methanol, filtered, and air-dried to yield 66 mg of material that melted at 226–227 °C. A mixture of this material and an authentic sample of unlabeled *myo*-inositol gave a mixed melting point of 227–228 °C. When trimethylsilylated according to the method of Sweeley et al. (1963), [using Sylon HTP (Supelco, Inc.)] and subjected to gas chromatography (using a 30 m × 0.25 mm SP2100 capillary column programmed at 6°/min from 120 to 250 °C) and chemical ionization mass spectroscopy (Finnegan 3200 system), the mass spectrum, when compared to that of unlabeled *myo*-inositol, indicated that the synthetic material is 98% monodeuterated.

For the preparation of [1-^3H]-*myo*-inositol by using the same procedure, precautions were taken (Naber, 1983) to trap all the $^3H_2$ liberated during the reduction with tritiated borohydride. To 15 mL of a solution (neutralized to pH 7.0) of *myo*-inosose-1, prepared as described above from 1.2 g of the phenylhydrazone, was added 15 mCi of sodium [^3H]-borohydride (350 mCi/mmol; New England Nuclear) in 2 mL of H_2O, and the reaction was stirred for 12 h at room temperature. At this point 0.2 g of unlabeled sodium borohydride in 5 mL of H_2O was added, and the mixture was stirred for

[1] Abbreviations: kat, katal (1 mol/s); inositol, *myo*-inositol; CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)-aminomethane; TLC, thin-layer chromatography; EDTA, ethylenediaminetetraacetic acid.

another 12 h at room temperature. The resulting product was purified as described for its deuterated analogue, and 37 mg of [1-^{3}H]-*myo*-inositol (365 mCi/mmol), mp 228 °C, was obtained. To ensure that tritium was attached to carbon and not to oxygen, water was added to the tritiated *myo*-inositol and then distilled out. No radioactivity was detected in the distillate.

Enzyme Assays. During enzyme purification the activity was assayed by the orcinol method (Reddy et al., 1981b) at 30 °C using 60 mM *myo*-inositol, 50 mM sodium acetate buffer, pH 6.0, 1 mM Fe(II), and 2 mM L-cysteine. However, most of the kinetic data reported here were obtained by following O_2 uptake with a Clark electrode and a Yellow Springs Instrument oxygen monitor (YSI Model 53) attached to an amplifier and a strip-chart recorder. Such assays were run at 30 °C in a total volume of 3 mL at the inositol concentration, pH, and buffer conditions given under Results. No Fe(II) or L-cysteine was present during these assays, so the background rate of O_2 uptake in the absence of enzyme is negligible. The reactions were initiated by the addition of a small aliquot of an enzyme solution that contained ca. 5 μg of inositol oxygenase and 30 μg of catalase; after a sample of the oxygenase was thawed for kinetic analysis, ca. 3 mg/mL crystalline bovine liver catalase (Sigma Chemical Co., 590 kat/kg) was immediately added and this enzyme stock solution stored (never longer than 1–2 h) on ice until use. The initial rate of disappearance of O_2 was followed for 1–2 min (during which time less than 10% of the O_2 reacts). Under these conditions the rate is linear with time. Enzyme activity is given in katals (kat[1]); 1 kat is equal to 1 mol of product formed or substrate reacted/s.

Deuterium and Tritium Isotope Effects. The deuterium isotope effect was determined by comparing the rate of O_2 uptake obtained with [1-^{2}H]-*myo*-inositol as substrate (k_D) with the rate that unlabeled *myo*-inositol reacts (k_H) under identical conditions. For each comparison the same thawed enzyme preparation or the same crude preparation was used.

The tritium isotope effect was measured by comparing the rate of O_2 uptake (k_H) to the rate of ^{3}H release to solvent (k_T) when [1-^{3}H]-*myo*-inositol is used as substrate. After the enzymic reaction had proceeded for 10–15 min (during which time the amount of O_2 uptake was monitored by using the O_2 electrode), it was terminated by the addition of 0.1 mL of concentrated sulfuric acid. The resulting mixture was distilled under atmospheric pressure, and aliquots (60–100 μL) of the distillate were counted for tritium by using the Scinti Versa scintillation cocktail (Fisher) and a Beckman LS7500 liquid scintillation spectrometer fitted with a 695010 data reduction accessory that calculates dpm's automatically. In typical enzymic reactions (3 mL) using 1–5 mM (1.1–5.5 mCi) [1-^{3}H]-*myo*-inositol, the 60–100-μL aliquots of distillate contained radioactivity that gave 500–900 dpm, reproducible to 20–30 dpm. In three different control experiments performed under the same conditions except that (a) boiled enzyme, (b) no enzyme, and (c) unlabeled *myo*-inositol were used, the distillate aliquots showed radioactivity indistinguishable from that of distilled water blanks (20–30 dpm).

Results

Enzyme Activity. In previous investigations (Reddy et al., 1981a,b; Reddy & Hamilton, 1981) the enzyme activity was measured by a colorimetric orcinol method for D-glucuronate following a 15-min incubation of enzyme and substrate in the presence of 1 mM Fe(II) and 2 mM cysteine at pH 6.0. In the absence of Fe(II) and cysteine, very little activity with purified enzyme preparations is detectable by such an assay.

Table I: Effect of Various Treatments on the Activity of Purified Inositol Oxygenase As Measured by the Orcinol Assay [with Fe(II) and Cysteine Present] or by the O_2 Uptake Assay [no Fe(II) or Cysteine Present]

enzyme treatment	specific activity (mkat/kg) orcinol assay[a,b]	specific activity (mkat/kg) O_2 uptake assay[a,c]
freshly prepared (never frozen) homogeneous oxygenase	20	1.3
enzyme frozen slowly at −20 °C and stored at −20 °C for 3 days	20	20
enzyme frozen rapidly in liquid N_2 and stored at −70 °C	20	0.3

[a] General reaction conditions: 30 °C; 60 mM sodium acetate buffer; pH 6.0; 60 mM *myo*-inositol; air-saturated solutions. [b] Reaction solutions also contained 15 μg/mL oxygenase, 1 mM Fe(II), and 2 mM L-cysteine, and the amount of D-glucuronate formed was determined after a 15-min reaction time. [c] Reaction solutions also contained 5 μg/mL oxygenase and 30 μg/mL catalase.

The activity could also be measured in the presence of Fe(II) and cysteine by following O_2 uptake with an O_2-sensitive electrode (Reddy et al., 1981a), but the high background rate due to autoxidation of the activators precluded accurate kinetic determinations of enzyme activity by this method.

In subsequent studies it was found that the homogeneous oxygenase is completely active even in the absence of Fe(II) and cysteine if it has been stored at −20 °C for days to weeks at pH 6.0 with 1 mM glutathione present. The results given in Table I indicate that it is very important how the enzyme is treated in order to observe this activity in the absence of activators. Thus, freshly prepared, but never frozen, samples of the homogeneous enzyme do not give this activity nor does enzyme that has been frozen rapidly in liquid N_2. To obtain the activity with no activators, the enzyme solution (approximately 0.5 mg/mL) in 50 mM sodium acetate buffer, pH 6.0, containing 50 mM KCl and 1 mM glutathione has to be frozen slowly over a period of a few hours (by placing it in a freezer at −20 °C) and then stored for several days at −20 °C. As the data in Table I show, the activity of the oxygenase measured by the orcinol assay with Fe(II) and cysteine present does not depend on how the purified enzyme is treated.

The enzyme activity measured by O_2 uptake in the absence of activators does, however, decrease with time unless catalase is present. In the absence of catalase, curvature in the O_2 uptake vs. time plots is evident even in the first 1–2 min, while in the presence of catalase (approximately 10 μg/mL) O_2 uptake is linear with time for at least 3 min, although some slowing down is detectable after 10 min. Catalase, however, does not affect the initial rate of the reaction. The ability of catalase to keep the enzyme active is not due to some generalized protein effect, because catalase cannot be replaced by other proteins such as bovine serum albumin. The results indicate that the inactivation in the absence of activators (Fe(II) and cysteine) is largely due to H_2O_2, which is known to be an inactivator of the enzyme (Reddy et al., 1981a). Indeed, when 0.55 nmol of oxygenase is treated with 1.6 nmol of H_2O_2, only 5% of the oxygenase activity remains even when measured in the presence of catalase. If catalase is present with the oxygenase prior to adding the H_2O_2, then the oxygenase is protected and 90% of the activity remains. Thus, catalase is a protector of the enzyme rather than an activator. The inactivation by H_2O_2 can, however, be essentially completely reversed with Fe(II) and cysteine. For example, when the above preparation of oxygenase with only 5% activity is assayed in the presence of 1 mM Fe(II) and 2 mM L-cysteine, it regains 96% of its original activity.

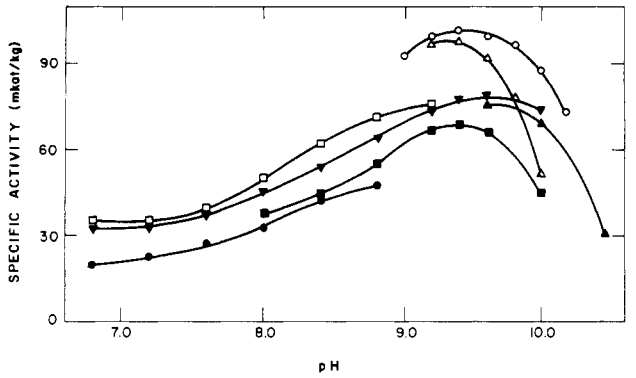

FIGURE 1: Effect of pH on the rate of the reaction catalyzed by homogeneous *myo*-inositol oxygenase when measured by the O_2 uptake method. Reaction conditions: volume 3 mL; 30 °C; 60 mM air-saturated buffer; 60 mM *myo*-inositol; 10–25 μg of inositol oxygenase that had been frozen and stored at −20 °C; 6 μg of catalase/μg of oxygenase; rates measured by O_2 uptake. (●) sodium phosphate; (■) borate; (▲) glycine; (▼) Tris-HCl; (△) carbonate; (○) CHES; (□) HEPES.

The H_2O_2 that leads to enzyme inactivation apparently arises from autoxidation of the glutathione used in storing the enzyme rather than by leakage of some intermediate in the enzyme-catalyzed reaction. In experiments where the formation of H_2O_2 was monitored (Kochli & von Wartburg, 1978) in the absence of catalase, it was found with the oxygenase present that the amount of H_2O_2 formed is the same in the absence or presence of the substrate *myo*-inositol, even though in the latter case the rate of O_2 uptake is at least 2 orders of magnitude faster.

Because of its protecting effects, in all subsequent kinetic experiments, catalase (6 μg/μg of oxygenase) was added to each stock oxygenase solution as soon as it was thawed and then this solution was used within 1–2 h. During this time the oxygenase so treated loses negligible activity when stored on ice. In the absence of catalase it loses approximately 30% of its activity in 2 h under similar conditions.

Explanations for many of the foregoing findings will be considered more extensively under Discussion, but the results suggest that Fe(II) and cysteine merely function to convert the enzyme from an inactive to an active state. Further evidence for this was obtained from an experiment in which catalytically inactive enzyme (in the absence of activators) was incubated with Fe(II) and cysteine and then the Fe(II) and cysteine were removed by dialysis. The resulting enzyme is now fully active catalytically in the absence of activators (experiment performed by M. Nozaki).

Regardless of any explanation for the activation of the enzyme on storage at −20 °C, the fact that this activated form can now be readily obtained and assayed in the absence of Fe(II) and cysteine allows one to examine the enzyme's kinetics, substrate specificity, pH effects, etc. with a much simpler assay system. Using the previous assay with Fe(II) and cysteine present, one was never sure whether the results being seen were due to effects on the activating system or were inherent characteristics of the oxygenase itself.

pH Effects and Apparent K_m for Inositol. Previously it had been reported (Reddy et al., 1981a) that the oxygenase in crude homogenates [where it does not require activation by Fe(II) and cysteine] has a pH maximum around pH 8 whereas the homogeneous enzyme, when assayed by the orcinol method with Fe(II) and cysteine present, has a maximum near pH 6. As the results in Figure 1 indicate, it is now clear that this difference is due to the effects of pH on the activating system rather than an inherent property of the enzyme itself. When assayed by the O_2 uptake method with no activators, the pH maximum of the purified enzyme is above pH 9, i.e., close to that of the enzyme in the crude homogenate. As noted previously, there is a marked buffer dependence, with reactions in CHES buffer giving the highest rates.

The results in Figure 1 illustrate the effects of pH on the maximum velocity because inositol is present in high concentrations. The apparent K_m for inositol when air-saturated solutions are used was also determined by the same assay method and at three different pH's (pH 7.4 and 8.0 in Tris-HCl and pH 9.4 in CHES buffer). The apparent K_m was found to be 5.0 ± 0.3 mM under all three conditions. Earlier Reddy et al. (1981a) obtained an identical value using acetate buffers from pH 4.5 to pH 7.0 and the orcinol method of assay with Fe(II) and cysteine present. Clearly, then, the apparent K_m is independent of pH, buffer, and method of assay.

Substrate Specificity. Previous investigations (Reddy et al., 1981a) had suggested that the enzyme is very specific for *myo*-inositol as substrate, but, because of the high background rate with the assay method used [O_2 uptake with Fe(II) and cysteine present], it could only be concluded that a number of other compounds were less than 15% as active as *myo*-inositol. Consequently, a survey of several potential substrates was repeated by using the current O_2 uptake assay without Fe(II) and cysteine. The results substantiate the earlier conclusion; no enzyme-catalyzed O_2 uptake was observed (2% of the rate obtained with *myo*-inositol could have been detected) with any of the following compounds at 5–50 mM concentrations in 60 mM acetate buffer, pH 6.0, or 60 mM Tris-HCl buffer, pH 7.4: D-glucose, D-xylose, xylitol, *cis*- and *trans*-cyclohexane-1,2-diol, L-inositol, *epi*-inositol, 2-*O*,*C*-methylene-*myo*-inositol (Posternak, 1944), 2-*C*-chloro- and bromomethyl-*myo*-inositol (Posternak, 1944; Giddey et al., 1974). Except for *epi*-inositol, none of the foregoing compounds was an effective inhibitor of *myo*-inositol oxidation either. (With *epi*-inositol, 49% inhibition was observed when it was present at 3.3 mM with 5 mM inositol as substrate at pH 7.4, Tris-HCl buffer, whereas less than 20% inhibition was seen with each of the other compounds under similar conditions.)

Inhibitors. It was reported previously (Reddy et al., 1981a) that the enzyme activity is decreased by a large number of different types of compounds when its activity is measured in the presence of Fe(II) and cysteine. By use of the present O_2 uptake assay in the absence of Fe(II) and cysteine, the enzyme's high sensitivity to oxidants (H_2O_2, ferricyanide, $FeCl_3$, $CuSO_4$, $HgCl_2$) and reductants (ferrocyanide) was confirmed. It is especially sensitive to ferricyanide; even with only 10 μM ferricyanide the enzyme is inhibited over 85%. The time course of inhibition by the reductant ferrocyanide is of some interest and is illustrated in Figure 2. Thus, the reaction starts off reasonably rapidly but soon stops; an explanation for his behavior will be given under Discussion.

Most of the other types of compounds reported by Reddy et al. (1981a) to be inhibitors when the enzyme was assayed by the orcinol method with Fe(II) and cysteine present were found not to be inhibitors when the enzyme was assayed by the O_2 uptake method with Fe(II) and cysteine absent. Thus, by this method less than 15% inhibition was obtained when each of the following compounds at 1 mM concentration was present in a reaction solution containing 60 mM Tris-HCl buffer, pH 7.4, 60 mM *myo*-inositol, 5 μg/mL inositol oxygenase that had been stored frozen at −20 °C, and 30 μg/mL catalase: metal-ion complexing agents (cyanide, azide, EDTA, oxalate, *o*-phenanthroline, α,α-bipyridine, 8-hydroxyquinoline,

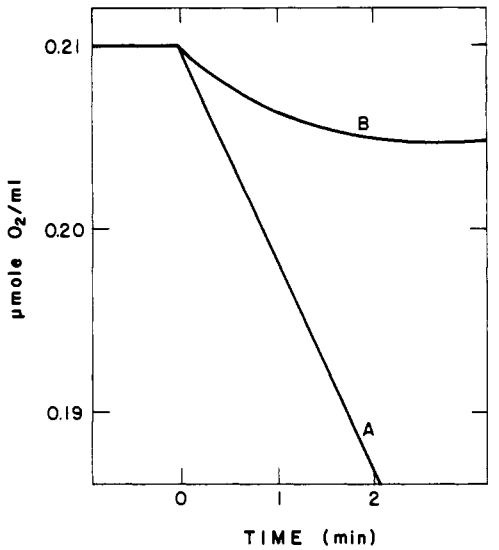

FIGURE 2: Inhibition of homogeneous *myo*-inositol oxygenase by ferrocyanide. Reaction conditions: volume 3 mL; 30 °C; 60 mM air-saturated Tris-HCl buffer; pH 7.4; 60 mM *myo*-inositol; 1 mM potassium ferrocyanide in B but no ferrocyanide in A; rates measured by O_2 uptake; reactions initiated at zero time by adding a small aliquot containing 15 μg of *myo*-inositol oxygenase (that had been stored frozen at −20 °C) and 90 μg of catalase.

xanthurenic acid, diethyldithiocarbamate), nucleotides (ATP, ADP, UTP, UDP), α-keto acids (oxalacetate, α-ketoglutarate, pyruvate, glyoxylate), barbiturates (barbital, phenobarbital), and thiol reagents [5,5′-dithiobis(2-nitrobenzoate), iodoacetamide, *N*-ethylmaleimide]. Presumably, then, these compounds affect the activating system rather than the actual oxygenase itself.

Inhibition by Potential Intermediates of the Oxygenase Reaction. L-*myo*-Inosose-1, the potential intermediate if the reaction proceeds by pathway A, Scheme I, is a potent competitive (vs. *myo*-inositol) inhibitor. At 30 °C in 60 mM air-saturated Tris-HCl buffer, pH 7.4, K_i was found to be 62 μM. It was also shown that, under these conditions, L-*myo*-inosose-1 (10 mM) is not a substrate for the enzyme (limit of detection, 2% of the *myo*-inositol rate).

The enzyme seems quite specific for L-*myo*-inosose-1 since two other isomers of this compound, namely, *myo*-inosose-2 and DL-*epi*-inosose-2, are much poorer inhibitors. Although extensive investigations with these compounds were not carried out, their K_i's were estimated to be approximately 5 and 1 mM, respectively, when measured under the same conditions as given above. Consequently, the strong inhibition by L-*myo*-inosose-1 is not a property of inososes in general.

A solution thought to contain D-glucodialdehyde (the potential intermediate if the enzymic reaction proceeds by pathway B, Scheme I) was prepared by oxidizing L-inositol with $NaIO_4$ (see Experimental Procedures). By analogy to similar reactions, D-glucodialdehyde should be the major product of this reaction because periodate is known to cleave *cis*-glycols more rapidly than *trans*-glycols (Bunton, 1965), and the structure of L-inositol is such that cleavage between either of its *cis*-glycol positions would give the same product, namely, D-glucodialdehyde. In order to minimize further cleavage a 0.5 molar ratio of periodate to L-inositol was used in the reaction. Because D-glucodialdehyde will exist as a complex mixture of hydrates and hemiacetals, no attempt was made to characterize the product. Assuming that the amount of dialdehyde formed is equal to the amount of $NaIO_4$ used, it was found that a solution calculated to be 1 mM in the dialdehyde causes only 5% inhibition (reaction conditions same as given above for the inhibition by L-*myo*-inosose-1 but with *myo*-inositol at 4.6 mM, i.e., near its K_m). In other experiments it was observed that up to 14 mM glutaryl dialdehyde causes no detectable inhibition of the enzyme under similar conditions. This lack of inhibition by dialdehydes implies that it must be just fortuitous that rat kidney inositol oxygenase binds to a dialdehyde affinity column (Koller & Koller, 1984).

Table II: Deuterium and Tritium Isotope Effects for the Oxidation of 1-Labeled *myo*-Inositols Catalyzed by Inositol Oxygenase[a]

buffer	pH	enzyme	[inositol] (mM)	k_H/k_D	k_H/k_T
Tris-HCl	7.4	purified	10	2.19	
Tris-HCl	7.4	purified	5	2.12	7.4
Tris-HCl	7.4	purified	2.5	2.11	
Tris-HCl	7.4	purified	2.0		7.6
Tris-HCl	7.4	purified	1.0	2.13	7.4
acetate	6.0	purified	1.0	2.07	7.4
CHES	9.2	purified	1.0	2.17	9.0
Tris-HCl	8.0	crude	5.0	2.09	7.5
Tris-HCl	8.0	crude	2.0	2.11	7.5

[a] Reaction conditions: volume 3 mL; 30 °C; 60 mM air-saturated buffer. For experiments with "purified" enzyme, 5–8 μg/mL homogeneous inositol oxygenase that had been stored at −20 °C and 30–50 μg/mL catalase were used. For experiments with crude enzyme, a 3 mg/mL concentration of the enzyme preparation obtained following the ammonium sulfate fractionation (Reddy et al., 1981b) was used. See Experimental Procedures for details concerning how k_H/k_D and k_H/k_T were obtained.

Deuterium and Tritium Isotope Effects. In Table II are summarized the results obtained from the oxidation of 1-labeled *myo*-inositols catalyzed by the oxygenase. It will be noted that the deuterium isotope effect (k_H/k_D) is invariant with conditions, being the same from pH 6 to pH 9.2, at several different inositol concentrations, and with both homogeneous and crude enzyme preparations. In the crude enzyme preparation the oxygenase is still part of an enzyme complex (Reddy et al., 1981b). The deuterium isotope effect is entirely due to an effect on the maximum velocity since the K_m for the reaction of the deuterated compound is identical (5.0 mM) with that for the unlabeled material, at least at pH 7.4 in Tris-HCl buffer.

The tritium isotope effect is also largely invariant with conditions although it appears to increase at pH 9.2. The experiment under these conditions was repeated several times so the difference is apparently real. The reproducibility for the k_H/k_T values is ±0.2.

Discussion

The finding that good enzymic activity for the virtually homogeneous *myo*-inositol oxygenase can be measured in the absence of the activators, Fe(II) and cysteine, is an important finding, because it has allowed considerable clarification of the enzyme's characteristics. Although the activation of the enzyme by careful freezing and storing at −20 °C seems somewhat bizarre, a reasonable explanation for this effect can be offered when it is considered along with the other results reported here and previously (Reddy et al., 1981a,b).

It now seems clear that there are two interconvertable forms of the enzyme, a catalytically active reduced form and a catalytically inactive oxidized form. Having Fe(II) and cysteine present apparently ensures that the enzyme is kept in the active reduced state. The observation that Fe(II) and cysteine can reverse the inactivation by H_2O_2, as well as the finding that, following incubation with an inactive enzyme, the activators can be removed and the enzyme now has full catalytic activity, is consistent with such a suggestion. When catalase is present the inactivation of the active form of the oxygenase by H_2O_2 is prevented, but catalase has no ability

to activate the inactive oxygenase. When present in crude homogenates, where it exists as part of an enzyme complex (Reddy et al., 1981b), the oxygenase is apparently resistant to oxidation because Fe(II) and cysteine are not required for full catalytic activity. During purification, however, when the oxygenase is separated from the complex, it apparently is readily oxidized and, in fact, is purified in the inactive state. Consequently, during purification it must be assayed with Fe(II) and cysteine present. When the purified enzyme solution is frozen slowly at −20 °C, it is expected that aqueous pockets, having a concentrated solution of enzyme, buffer, KCl, and glutathione, will be formed in the ice (Pincock, 1969). It that is so, then it seems reasonable that, over time, the oxidized enzyme would get reduced by the glutathione and thus activated as observed. On the other hand, when the enzyme is frozen rapidly in liquid N_2 and kept at −70 °C, the aqueous pockets would be much smaller, and possibly nonexistent, so reduction and activation of the enzyme would be much less likely to occur, again as observed.

With the ability to assay the pure enzyme's activity in the absence of activators, it was possible to show that the pH–rate profile for the purified enzyme (Figure 1) does not have a maximum at pH 6 as was reported previously when it was assayed in the presence of Fe(II) and cysteine, but rather has a profile (maximum above pH 9) similar to that of the enzyme in the native complex (Reddy et al., 1981a). Therefore, the earlier results are due to an effect of pH on the activating system rather than on the enzymic reaction itself. The inability of Fe(II) and cysteine to activate at higher pH values is probably due to their ease of autoxidation under such conditions. The effect of pH on the enzymic rate is entirely due to an effect on the maximum velocity since the apparent K_m for inositol (air atmosphere) remains constant at 5.0 mM over the entire pH range.

By use of the new assay system, the high specificity of the enzyme for *myo*-inositol as substrate was confirmed; no other potential substrate reacts at even 2% of the *myo*-inositol rate. The sensitivity of the enzyme to oxidants and reductants was also confirmed, but many other compounds, reported previously (Reddy et al., 1981a) to inhibit the enzyme when it is assayed with Fe(II) and cysteine present, were found to have no effect when the enzyme is assayed in the absence of these activators. Many of these are metal-ion complexing agents or thiol reagents, so it is perhaps not too surprising that they would affect the activating system.

The high sensitivity of the oxygenase to oxidants, such as ferricyanide, is a further indication that it is a reduced form of the enzyme that is the catalytically active form. On the other hand, the characteristics of the inhibition by ferrocyanide (Figure 2) imply that, during catalysis, highly oxidized forms of the enzyme are involved as intermediates. The observation, that the enzymic reaction starts off reasonably rapidly in the presence of ferrocyanide but soon stops, suggests that ferrocyanide is trapping some intermediate during catalytic turnover, and the only type of intermediate which it seems reasonable that a reductant, such as ferrocyanide, could trap is a highly oxidized species. What type of structure such a species might have will have to await further experimentation.

The potent competitive inhibition of the oxygenase by L-*myo*-inosose-1 is an initial indication that this compound may be an intermediate in the reaction. The observations, that the enzyme shows high specificity for this derivative (other inososes are much poorer inhibitors) and that it binds almost 2 orders of magnitude more tightly than the substrate *myo*-inositol itself (K_i = 62 μM, K_m = 5.0 mM), are considered good evidence for this possibility. The lack of inhibition by a solution thought to contain the putative dialdehyde intermediate is consistent with the above conclusion, although it cannot be considered strong evidence because the dialdehyde was not characterized.

Stronger evidence that the reaction proceeds by a mechanism such as pathway A rather than the alternative pathway B (Scheme I) is the fact that a relatively large tritium isotope effect is observed for the reaction when *myo*-inositol is labeled with tritium in the 1-position. The deuterium isotope effect can be explained by either pathway, but because of the way it is measured, the observation of a tritium isotope effect is only consistent with the reaction occurring by pathway A or a closely related pathway. The main difference in the two pathways is that the carbon–hydrogen bond in question is broken in the first step of the mechanism in pathway A, whereas it is not broken until the final step in pathway B. Because tritium isotope effects are measured by competition methods (only a small amount of labeled substrate and a large amount of unlabeled substrate), an absolute requirement in order to observe a tritium isotope effect is that all intermediates, up to and including that which loses the tritium, must be in equilibrium (or at least partial equilibrium) with the substrate free in solution. That would certainly be possible if the reaction occurs by pathway A, because the step involving the cleavage of the carbon–hydrogen bond would probably only be preceded by binding of the substrate to the enzyme. However, it is very unlikely that the intermediate that loses the hydrogen in pathway B could be in equilibrium with *myo*-inositol, because one expects that the first step in this pathway would be irreversible. As has been discussed elsewhere (Hamilton, 1974), reactions of O_2 with organic compounds are considerably exothermic, so the possibility that the first step of pathway B could be reversible is very remote.

In summary then, the data presented here strongly favor pathway A rather than pathway B for the *myo*-inositol oxygenase-catalyzed reaction. Minor variations to the specific pathway illustrated in Scheme I would also be consistent with the tritium isotope data, but the strong and specific inhibition by L-*myo*-inosose-1 suggests that it is a real enzyme-bound intermediate. Thus, alternative mechanisms involving the conversion of *myo*-inositol directly to the peroxide adduct that cleaves to glucuronate seem less likely. The intermediate L-*myo*-inosose-1 would, however, have only a fleeting existence because the observation of a kinetic deuterium isotope effect for the reaction indicates that the step involving cleavage of the carbon–hydrogen bond is at least partially rate determining for the overall reaction. It is probably not completely rate determining because k_H/k_D (Table II) is too small relative to k_H/k_T. According to the Swain relationship (Swain et al., 1958; Northrop, 1976), k_H/k_T should equal $(k_H/k_D)^{1.442}$ if they are both intrinsic isotope effects, and clearly k_H/k_T is considerably larger than that.

If the reaction proceeds as in pathway A, then it seems likely that the function of the enzyme-bound iron is connected with catalysis of the first step rather than of subsequent ones. Many reactions analogous to these latter steps have been studied in detail (Fraser & Hamilton, 1982; Hamilton, 1974), and there is no indication that metal ions either accelerate or are required for such reactions. Therefore, one of the remaining interesting questions with this enzyme is how nonheme iron catalyzes the oxidation of an alcohol to a ketone with O_2 as oxidant. Copper-containing oxidases are known to catalyze such reactions (Hamilton, 1981; Hamilton et al., 1978), but as far as the present authors are aware, no other nonheme iron enzyme does so. It will be of interest to determine whether the

mechanism of the reaction catalyzed by this iron enzyme is similar to that of the reaction catalyzed by the copper enzyme, where higher valence states of the copper are involved. The inhibition of *myo*-inositol oxygenase by reductants such as ferrocyanide (Figure 2) suggests that may be the case.

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Registry No. D_2, 7782-39-0; T_2, 10028-17-8; [1-^{2}H]inositol, 104323-38-8; [1-^{3}H]inositol, 104335-63-9; inositol, 87-89-8; L-*myo*-inosose-1-phenylhydrazone, 22263-78-1; L-*myo*-inosose-1, 56816-02-5; *myo*-inositol oxygenase, 9029-59-8.

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