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AN OXYGEN-18 TRACER INVESTIGATION OF THE MECHANISM OF myo-INOSITOL OXYGENASE

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SUMMARY: In the conversion of myo-inositol to D-glucuronic acid catalyzed by myo-inositol oxygenase only one atom of ^{18}O from $^{18}O_2$ is incorporated into the product, and it is found exclusively in the carboxyl group. Control experiments indicate that under the reaction conditions no exchange of solvent oxygens with D-glucuronate occurs. To avoid exchange during isolation and analysis the oxygenase product was enzymically reduced to L-gulonate and isolated in that form. The results eliminate one possible mechanism for the oxygenase reaction, but are consistent with two others which seem chemically reasonable.

myo-Inositol oxygenase (EC 1.13.99.1), which catalyzes the conversion of myo-inositol into D-glucuronic acid, was initially characterized by Charalampous in the late 1950's (1,2). The enzyme is present in both plants (3,4) and animals (1,2) but in the latter is found only in kidneys (5). In his initial investigations with the rat kidney enzyme, Charalampous carried out the reaction using $^{18}O_2$, isolated the D-glucuronic acid, converted it to CO_2, and analyzed the CO_2 for its ^{18}O content (6). From the results obtained it was concluded that the isolated D-glucuronate contained one atom of oxygen which was derived from O_2. Due to the method of analysis it was not possible to conclude definitely which oxygen atom of the D-glucuronate contained the label although it was presumed to be in the carboxylate. A further complication in Charalampous' experiments is that the carbonyl oxygen of aldehydes exchanges readily with water, and thus any ^{18}O which might have been present in the aldehyde group of the enzymically formed D-glucuronate could have been exchanged out during its isolation by ion exchange chromatography.

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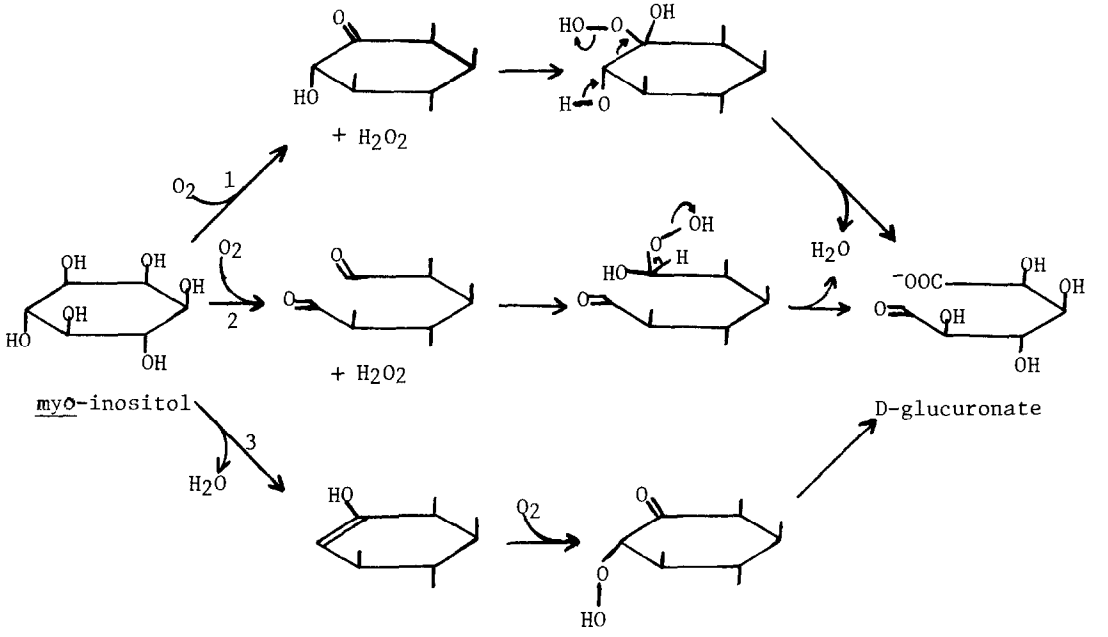
As part of our continuing interest in the mechanisms of enzymic oxidation-reduction reactions (7,8), we have recently purified the hog kidney inositol oxygenase to homogeneity (9), and have been studying various aspects of its mechanism. For the overall reaction there are three general pathways which appear reasonable from a mechanistic standpoint. These are outlined in Scheme 1. If the reaction proceeds by either pathways 1 or 2 then only one atom of



Scheme 1

oxygen from O_2 should end up in the D-glucuronate, and it should specifically be in the carboxylate. However, if the reaction proceeds by pathway 3, then one atom of oxygen from O_2 will be present in the aldehyde group, and the other could end up in either the carboxylate or the solvent depending on the details of the conversion of the α-hydroperoxy ketone to D-glucuronate.

In order to distinguish pathways 1 and 2 from pathway 3, and also in order to remove the ambiguities associated with the earlier work (6), we have directly determined what specific groups of the enzymic product obtain an isotopic label from $^{18}O_2$. To avoid the possibility of the aldehyde group of D-glucuronate exchanging with solvent, we have employed in these experiments a partially purified enzyme preparation which not only contained the oxygenase but also had D-glucuronate reductase (EC 1.1.1.19) activity. Thus, in the presence

of excess NADPH the D-glucuronate formed in the oxygenase reaction is immediately converted to L-gulonate which does not exchange oxygens with the solvent under the conditions employed. To determine the positions labeled with isotopic oxygen from $^{18}O_2$ the L-gulonate was isolated, trimethylsilylated and analyzed directly by mass spectral techniques.

EXPERIMENTAL PROCEDURE

MATERIALS: Unless otherwise noted, commercial materials were used as received. Isotopically enriched $^{18}O_2$ gas of 90% isotopic purity was obtained from Merck Isotopes, and 20% $H_2{}^{18}O$ from Bio-Rad. L-Gulono-γ-lactone (mp. 183-4°) was obtained as a gift from ICI Americas.

ENZYME ASSAYS: The oxygenase activity was determined by measuring the amount of D-glucuronate formed using the orcinol method (1,9). Reaction mixtures (2.0 ml) containing 50 mM sodium phosphate buffer, pH 7.2, 2.0 mM cysteine, 1.0 mM ferrous ammonium sulfate, 60 mM myo-inositol, and appropriate quantities of enzyme were incubated with shaking in contact with an air atmosphere at 30° for 15 min., then quenched with 0.1 ml of trichloroacetic acid, centrifuged, treated with the orcinol reagent as previousiy described (1), and analyzed at 660 nM. D-glucuronate reductase activity was determined spectrophotometrically at 30° by monitoring the oxidation of NADPH at 340 nM. Reaction solutions (1.0 ml) contained 50 mM sodium phosphate buffer, pH 7.2, 0.2 mM NADPH, 10 mM D-glucuronate and appropriate quantities of enzyme. Protein concentrations were determined by the method of Warburg and Christian (10), and enzyme activities are expressed in katals/kg (1 katal is equal to 1 mole/sec.).

ENZYME PREPARATION: The oxygenase and reductase were partially purified from hog kidney by following the initial steps in a procedure recently developed in this laboratory for obtaining the homogeneous oxygenase (9). The steps involved homogenization, centrifugation and ammonium sulfate fractionation (the enzymes precipitate between 30 and 45% saturation). For the experiments reported here the oxygenase activity was 18 μkat/kg and the reductase activity 1.1 mkat/kg.

ENZYMIC EXPERIMENTS UTILIZING $^{18}O_2$: These were carried out in a 100 ml 3-necked flask, one exit fitted with a rubber septum, another with a pressure equalizing stoppered funnel, and the third with an adaptor and 3-way stopcock for connecting either to a vacuum pump or to the $^{18}O_2$ sample. The enzyme preparation (10 ml) containing 26 mg/ml protein, 25 mM sodium phosphate buffer, pH 7.2, and 1 mM glutathione was placed in the flask, and 1.2 ml of 100 mM myo-inositol in the funnel. After evacuation, enough $^{18}O_2$ was introduced to give a final pressure of approximately one-third to one-half atmosphere. Just prior to initiating the reaction by adding the inositol, 0.6 ml of a solution containing 40 mM cysteine and 20 mM Fe(II) was added, and 0.2 ml of 120 mM NADPH was injected immediately following initiation. After stirring for 20 min. at room temperature, a sample of the gas phase was removed by gas tight syringe for analysis of the $^{18}O_2$ content, and the enzymic reaction was terminated by boiling for 5 min. To ensure complete protein removal, the solution was treated twice with an equal

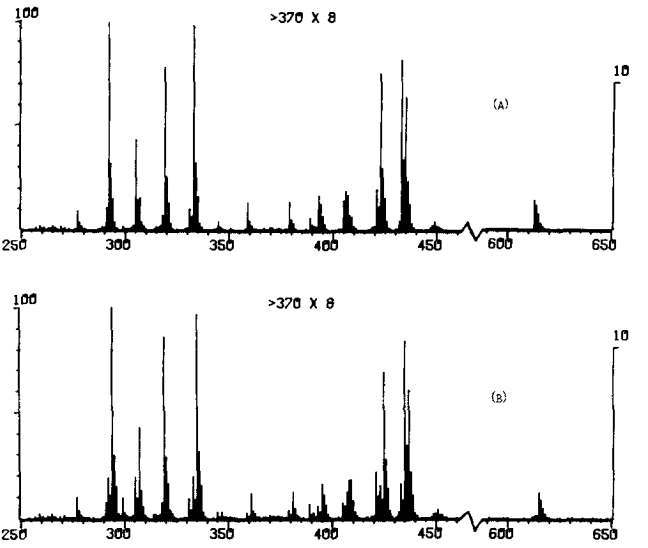


Figure 1. The partial mass spectrum of unlabeled hexakis-O-(trimethylsilyl)-L-gulonic acid (a), and (b) that of the trimethylsilylated product isolated from the enzymic reactions performed using 90% $^{18}O_2$.

volume of chloroform, then centrifuged and the aqueous layer filtered through an Amicon ultrafiltration apparatus. A 3.0 ml aliquot of the filtrate was reduced to 0.2 ml by evaporation under vacuum, treated with 6.0 ml of ethanol, and stored overnight at -10°. The solid was collected, dried well under vacuum, trimethylsilylated (using Sylon HTP obtained from Supelco, Inc.) according to the procedure of Sweeley et al. (11), and analyzed using a Finigan 3200 gas chromatograph-mass spectrometer equipped with a Finigan 6000 data system. Separation of the components was achieved isothermally at 160° using a 5 ft. by 1/8 inch glass column packed with 3% OV-17 (detector and injector temperatures were 250°). The ions were produced by electron impact (70 electron volts) and analyzed with a quadrapole mass analyzer.

PREPARATION OF L-GULONATE WITH ^{18}O IN THE CARBOXYLATE GROUP: L-Gulono-γ-lactone and equimolar amount of base were dissolved in $H_2{}^{18}O$ and left to sit overnight at 37°. The solution was neutralized, concentrated by evaporation, and the solid L-gulonate isolated, dried, and analyzed by the procedure outlined above.

POSSIBLE D-GLUCURONATE EXCHANGE WITH $H_2{}^{18}O$: D-Glucuronate (10 mg) was dissolved at room temperature in 0.5 ml $H_2{}^{18}O$ buffered at pH 7.1 with 50 mM sodium phosphate. At various times a 0.1 ml aliquot was removed and treated with 1 mg $NaBH_4$. Excess $NaBH_4$ was reacted by adding 0.1 ml of 1 N HCl and borate was removed by passing the solution through a mixed ion exchange resin. After evaporating to dryness the L-gulonate was analyzed for its ^{18}O content as previously described.

RESULTS

Shown in Figure 1(a) is the partial mass spectrum of an unlabeled authentic sample of hexakis-O-(trimethylsilyl)-L-gulonic acid, and in

Fig. 1(b) the spectrum obtained from the trimethylsilylated product isolated from the enzymic reactions performed using 90% $^{18}O_2$. It will be seen that most, but not all, of the peaks in the second spectrum are shifted by 2 units. This includes the peak at m/z = 613 which corresponds to the molecular ion(m/z = 628) minus a methyl group. From this qualitative observation, it is thus clear that the L-gulonic acid formed from inositol under the present conditions contains only one atom of oxygen derived from O_2. To determine whether this result might be due to the intermediate D-glucuronate exchanging its aldehyde oxygen with solvent under the reaction conditions, D-glucuronate was incubated with $H_2{}^{18}O$ for up to 20 min (the time of the enzymic reaction), then reduced to L-gulonate with $NaBH_4$, and its ^{18}O content analyzed. The spectrum obtained (not illustrated) is indistinguishable from that in Fig. 1(a), i.e., no exchange was observed. Since D-glucuronate reductase and excess NADPH were present in the enzymic reaction, the intermediate D-glucuronate would have existed for much less than 20 min. Therefore, it can be safely concluded that, in the enzymic experiments, none of the ^{18}O label present in the D-glucuronate would have exchanged out. Consequently, the fact that only one atom of ^{18}O is found in the isolated L-gulonate indicates that, in the oxygenase-catalyzed conversion of inositol, only one atom of oxygen from O_2 is incorporated into the D-glucuronate.

The fragment peaks that are shifted by two units in the spectrum of Fig. 1(b) relative to those in Fig. 1(a) give an initial strong indication that the ^{18}O is in the carboxyl group of the L-gulonic acid; each of the shifted peaks can be readily rationalized in terms of a structure involving the carboxyl function. That the ^{18}O is in the carboxyl group was confirmed when the spectrum was taken of a trimethylsilylated authentic sample of carboxyl labeled L-gulonic acid which had been prepared by hydrolysis of L-gulono-γ-lactone in $H_2{}^{18}O$. Exactly the same peaks are shifted in the spectrum (not illustrated) obtained from the authentic sample as are shifted in the spectrum (Fig. 1(b) obtained from the enzymic product. Especially notable is that the major peak at m/z = 319, which arises from a four carbon fragment that does not contain the carboxyl group, is not shifted in either spectrum.

As indicated earlier, a visual examination of the spectra (Fig. 1) indicates qualitatively that only one atom of oxygen from $^{18}O_2$ is incorporated into the enzymic product. A quantitative analysis of the data (for details see reference 12) indicates that 0.97 atoms are

incorporated. This was calculated from the mass spectrometrically determined ^{18}O content in the $^{18}O_2$ used in the enzymic experiments, and from the intensities of several of the shifted peaks in Fig. 1(b) relative to those in Fig. 1(a). Essentially the same number is obtained from an analysis using each of the following pairs of peaks m/z : 613, 615; 433, 435; 423, 425; 333, 335; and 292, 294. In some cases a small correction was made (12) when there are overlapping peaks.

DISCUSSION

The results reported here establish that only one oxygen from O_2 is incorporated into the D-glucuronate formed from myo-inositol in the oxygenase-catalyzed reaction, and that it is specifically incorporated into the carboxylate group. Under the enzymic reaction conditions, it was shown that no exchange of the D-glucuronate with solvent occurs. Any possible exchange during the isolation, and during analysis of the ^{18}O content of the product, was avoided by enzymically converting the D-glucuronate to L-gulonate as soon as it is formed.

As a result of this research, pathway 3, Scheme 1 is eliminated as a possible mechanism for the enzymic reaction. However, the data are consistent with both pathways 1 and 2. Further work is continuing to distinguish between these alternatives.

Since only one atom of oxygen from O_2 is incorporated into the product even though the overall enzymic reaction is a four electron oxidation, inositol oxygenase should now be included in that group of enzymes termed internal monooxygenases (13). As such, it should be classified in the EC 1.13.12 group.

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