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Tracing Oxygen Metabolism by Use of Positron Emitter Oxygen-15

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Oxygen, an essential molecule for life, is utilized not only for cellular respiration but also for biosynthesis and metabolism of various important biomolecules such as steroids, eicosanoids, and neuroactive substances. Since the oxygenases, oxygen-fixating enzymes, were found in 1950s, only stable isotopes (^{17}O and ^{18}O) have been utilized as a tracer for demonstration of oxygen incorporation into organic substances. This stable isotopic method is established, but is hardly applicable to complicated (crude and multi-cellular) systems. Therefore, we here developed a novel radiotracer technique for oxygen metabolism that employs the positron emitter $^{15}O_2$, whose physical half-life is 2.07 min. In a model reaction with metapyrocatechase, one of the well-known dioxygenases, the substrate catechol was converted to the radioactive product which was identified as α-hydroxymuconic ϵ-semialdehyde by a very sensitive LC-radio-UV-MS combined method.

Since oxygenase, a molecular oxygen-fixating enzyme, was found by Mason et al. (1) and Hayaishi et al. (2) independently in the 1950s, a multitude of biochemical studies related to oxygen metabolism, oxygen radicals, and biological oxidations have been performed (3-5). The oxygenases were purified and well characterized, and some of them were crystallized, and their peptide and cDNA structures clarified (3-5). There are a number of studies that have provided useful informations on the kinetics of oxygen and substrate interaction including those that identified the oxygenated ternary complex of enzyme molecules (3-5). A number of oxygenases require heme iron or nonheme iron as a prosthetic group. The metabolic or biosynthetic pathways of valuable bioactive substances such as neurotransmitter amines, steroids, and eicosanoids have been demonstrated to be mainly catalyzed by a variety of mono- and di-oxygenases. Detoxication of various drugs and foreign substances is carried out by the action of cytochrome P450, a member of monooxygenase group. Nitric oxide and carbon monoxide, both of which are bioactive substances that have attracted many researchers' interest in this decade, are also produced by the catalysis of monooxygenases.

Previously, evidence for oxygenase reactions was mostly obtained by use of stable oxygen isotopes, ^{17}O or ^{18}O, in such a way that the purified product was analyzed by mass spectrometry (1-5). However, this method is hardly applicable to analyze and identify an unknown substrate(s) and/or unknown product(s) in crude systems, although Hayaishi et al. (6) determined %incorporation of molecular oxygen into organic substances (totally unknown compositions) in microorganisms by using stable isotopic method. For studies on oxygen metabolism, especially unknown oxygenase reactions, the development of oxygen radiotracer techniques is indispensable. Likewise, the nature of the oxygen sensor for hypoxemia, which is located in the carotid body cells, chromaffin cells of the adrenal medulla, and some neurons in the brain can be studied with a radiotracer of oxygen molecules. Unfortunately, oxygen isotopes lack a suitable β^--emitter, unlike ^3H, ^{14}C, ^{32}P, and ^{35}S used routinely in biochemistry. The only available oxygen β^--emitter, ^{19}O, has a half-life of 27.1 sec. Instead, we have access to a positron emitter, ^{15}O, that has a physical half-life of 2.07 min and has been used for positron emission tomography (PET). The main problem encountered when performing experiments using molecules with such a short-lived radionuclides is related to time. Therefore, in the present

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Abbreviations used: LC-radio-UV-MS, liquid chromatography-mass spectrometry with radio- and ultraviolet absorption-detections; LC-MS, liquid chromatography-mass spectrometry; KPB, potassium phosphate buffer.

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study, we utilized a very sensitive radio LC-MS method (7) to analyze the reaction product with radiodetection within 7 min and succeeded to follow the reaction of one of the typical dioxygenases with a high turnover number, metapyrocatechase (catechol:oxygen 2,3-oxidoreductase, EC 1.13.1.2).

EXPERIMENTAL PROCEDURES

A schematic drawing of the experimental setup for the $^{15}O_2$-oxygenase reaction is shown in Fig. 1. Oxygen-15 was produced by the $^{14}N(d, n)^{15}O$ nuclear reaction by irradiation of the $^{14}N_2$ gas target with 9 MeV deutrons in a Scanditronix MC-17 Cyclotron at the Uppsala University PET Centre. $^{15}O_2$ was transported via Teflon tubing (ca. 30 m) to the reaction vial (injection vial, Apoteksbolaget, Sweden) through a septum by the flow of 0.5% O_2/99.5% N_2 gas mixture.

Prior to the introduction of radioactive oxygen, all solutions except the enzyme solution and vessels were equilibrated with nitrogen gas. Metapyrocatechase solution dissolved in acetone-containing (10%, v/v) potassium phosphate buffer (50 mM, pH 7.4) was gently bubbled with helium. The standard reaction mixture (total volume, 5.0 ml) contained as final concentrations 50 mM potassium phosphate buffer (KPB, pH7.4), 5 mM catechol (1% ethanol), and the enzyme (activity, 0.62 μmol/min at normal oxygen partial pressure, corresponding to 21 μg of protein). In the standard assay at room temperature (22-24°C), KPB + catechol solution was bubbled with nitrogen gas through the tubing from the target to the reaction vessel. Then, delivery of $^{15}O_2$ was started. After the radioactive counts had reached saturation in the vessel, the enzyme solution was added through the septum to start the reaction. The enzyme reaction was performed for 4 min and analyzed by LC-radio-UV-MS according to the conditions described below. The LC-MS condition was established by use of the product generated by the enzyme under non-radioactive conditions. One-tenth volume of 2 M HCl was added to the mixture to terminate the reaction. An aliquot of the mixture was taken through the septum, and, after filtration through a 0.22-μm pore-size filter (Millipore), it was tranferred into the LC fraction tube for LC-radio-UV-MS analysis. HPLC conditions: A Beckman 126 pump delivered a flow of 1.0 ml/min to a column (I.D.4.6 × 100 mm) of Kromasil C18 (5 μm) held at 40°C. Mobile phases were (A) 3 mM formic acid in Nanopure water (Barnsted) and (B) 3 mM formic acid in acetonitrile. A linear gradient from 2.5-98.5%B in 6 min was used. Detection: A 1:50 postcolumn split delivered 98% of the flow to a Beckman 166 variable wavelength UV detector (360 nm) and then to a Bioscan Flow-Count β^+-detector. The remaining 2% was delivered to a Fisions VG Quattro mass spectrometer equipped with pneumatically assisted electrospray, run in negative mode. The spray capillary was held at 2.5 kV, and the source temperature at 80°C. Selected ion recording was performed in two channels: Channel 1, m/z 109 [M-1], Dwell 0.1 Secs, Cone 30 V; Channel 2, m/z 141 [M-1], Dwell 1.0, Cone 20 V.

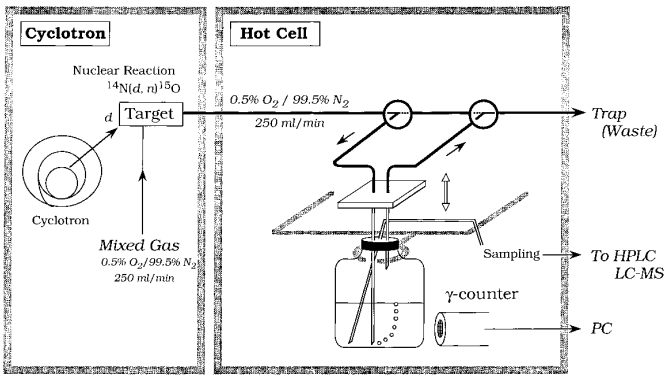

FIG. 1. Schematic drawing of the experimental setup for the $^{15}O_2$-oxygenase reaction. Oxygen-15 was produced by the $^{14}N(d, n)^{15}O$ nuclear reaction by irradiation of the $^{14}N_2$ gas target with 9 MeV deutrons in a Scanditronix MC-17 Cyclotron at the Uppsala University PET Centre. $^{15}O_2$ was transported via Teflon tubing (ca. 30 m) to the reaction vial (injection vial, Apoteksbolaget, Sweden) through a septum by the flow of 0.5% O_2/99.5% N_2 gas mixture. Remote-controlled elevators and two two-way electromagnetic valves made in the mechanical house of PET Centre were installed in the reaction apparatus. A γ-counting unit was situated close to the reaction vessel and was connected to a PC to monitor the incorporation of radioactive oxygen into the system.

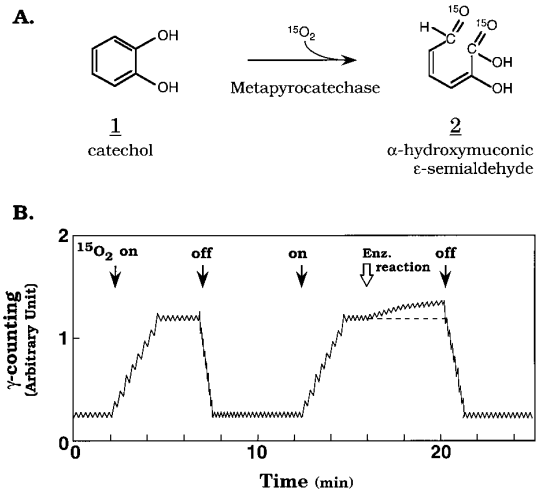

FIG. 2. Reaction scheme of metapyrocatechase with $^{15}O_2$ (A), and a representative pattern of γ-counting in introduction of $^{15}O_2$ into the reaction vessel and during the enzymatic reaction (B).

RESULTS AND DISCUSSION

In Fig. 1, a rough sketch of the experimental setup is shown. In this setup, the flow rate was optimized for trapping the maximum amount of radioactivity in the reaction vessel. With a flow rate from 250 ml to 750 ml/min, the maximum count was obtained in the reaction vessel; and it reached equilibrium within 3 min after the start of flow from the target. To afford the lowest possible degeneration of the enzyme due to vigorous bubbling, we selected the flow rate of 250 ml/min. As the reaction proceeded after addition of the enzyme to the reaction mixture that had already been equilibrated with the flow of radioactive oxygen, the radioactivity increased in the reaction vessel (Fig. 2), possibly because of the incorporation of oxygen molecules into the product and/or adducts with the enzyme.

After optimization of the enzyme reaction, the product was analyzed by the LC-radio-UV-MS system. As shown in Fig. 3, simultaneous multi-detection, i.e., radioactivity, UV absorption at 360 nm, selected ion recording (SIR) for the enzyme product (141.0), and for

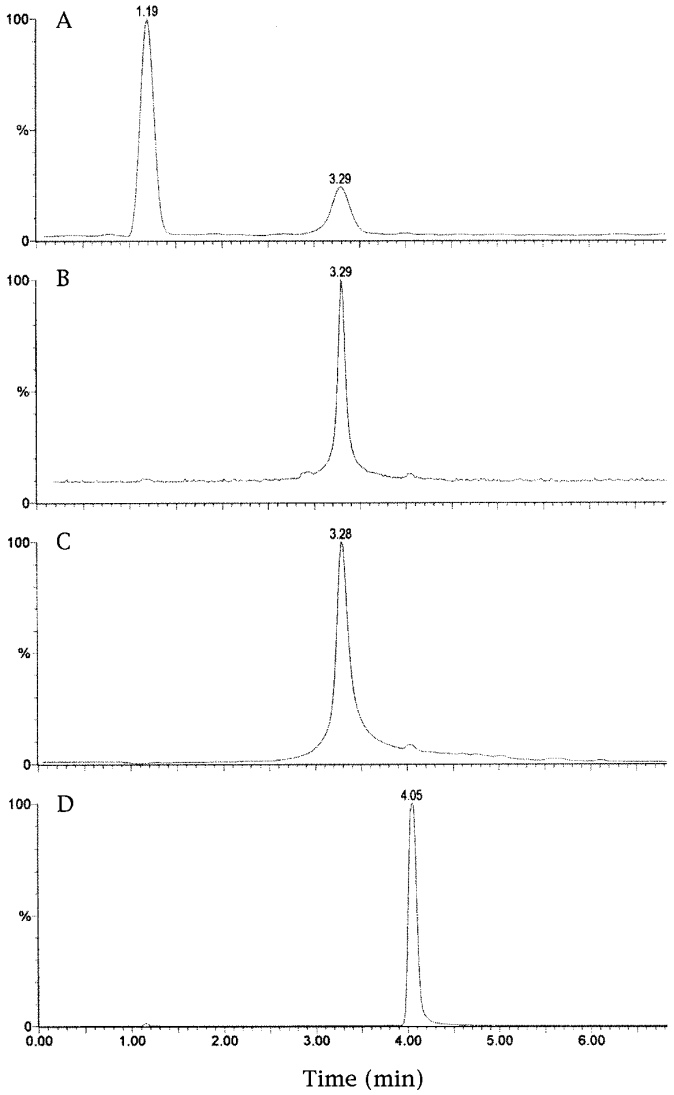

FIG. 3. Typical LC-radio-UV-MS integrated traces of the reaction. The reaction was performed for 4 min and analyzed by LC-radio-UV-MS according to the conditions described under Experimental Procedures. The LC-MS condition was established by use of the product generated by the enzyme under non-radioactive conditions. (A) Radioactivity; (B) UV absorption at 360 nm (375 nm is the maximum absorption wavelength of α-hydroxymuconic ϵ-semialdehyde, the product); (C) selected ion recording at MS 141.0 for α-hydroxymuconic ϵ-semialdehyde; (D) selected ion recording at MS 109.0 for catechol. The numbers above the peaks are the retention times. The decay of ^{15}O was not corrected.

the substrate catechol (109.0), clearly demonstrated the identification of the radioactive product after a 4-min reaction. When the time course was followed, the reaction proceeded almost linearly for 2 min and reached its plateau at around 4 min. The radioactivity, UV absorption at 360 nm, and MS141.0 showed peaks with essentially the same retention time. The reaction was reproducible with the same amount of enzyme, and seemed to be dependent on the enzyme amount in some range (Fig. 4A, B, and C). When the enzyme was omitted and just the buffer for the enzyme solution was added to the reaction mixture instead, there were no peaks at all in any of the three detection systems (bottom traces in Fig. 4A, B, and C). Free radioactive oxygen molecules dissolved in the reaction mixture was stacked on the column at this pH. The results were also the same when the substrate was omitted from the reaction mixture (data not shown).

As shown in Figs. 3 and 4, there were two radioactive peaks, one at the position corresponding well to the authentic product (α-hydroxymuconic ϵ-semialdehyde, *2*) and the other at the void volume. Since the radiochromatogram was not corrected for decay, almost double the amount of the product *2* shown was actually present. Therefore, the ratio of the radioactivity between the real product *2* and the void-volume compound was 0.6:1. UV and MS peaks were not necessarily corrected since the percentage of ^{15}O-containing product was less than 0.01% of the total amount of the product, i.e., the dilution factor by ^{16}O was more than 10,000. The addition of an anti-oxidant such as ascorbic acid (final concentration, 2 mM) or cysteine (1 mM) had little effect on the production of this early radioactive peak. Furthermore, the addition of ferrous ammonium sulfate (1 mM) (8) had no effect. A portion of the reaction mixture, either after the reaction had been stopped by addition of 0.2 M (final conc.) HCl or directly after the reaction without being quenched by acid, was immediately separated into high molecular weight and low molecular weight fractions by use of a PD-10 (Sephadex G-25) column, and the radioactivities of the fractions were counted. As a result, most of the radioactivity was in the low molecular weight fraction, and this fraction contained both void volume peak and the product peak found by LC-radio-UV-MS analysis (data not shown). The addition of an enzyme inhibitor (0.1 mM α-naphtoquinone or 0.1 mM *m*-phenanthroline) (8) to the standard reaction mixture reduced the formation of both radioactive peaks by more than 85%.

The radioactive peak at the void volume did not give any remarkable mass peak by LC-MS, had a low molecular weight, and had no particular UV peaks at 280 and 360 nm. However, this peak was only seen after the mixing of the three components, i.e., substrate, oxygen, and enzyme. None of the various combinations of two of the three components gave the product peak nor this void radioactive peak. Semi-proportional production of this radioactive peak to the radioactive enzyme product when the enzyme amount was changed (Fig. 4A) tempts us to speculate that the peak could be an enzyme peptide fragment bearing radioactive oxygen or perhaps a ferrous-oxygen complex resulting from the enzyme reaction and degradation of the active site by irradiation, since the active site of the enzyme consists of ferrous iron and amino acid residues. The true iden-

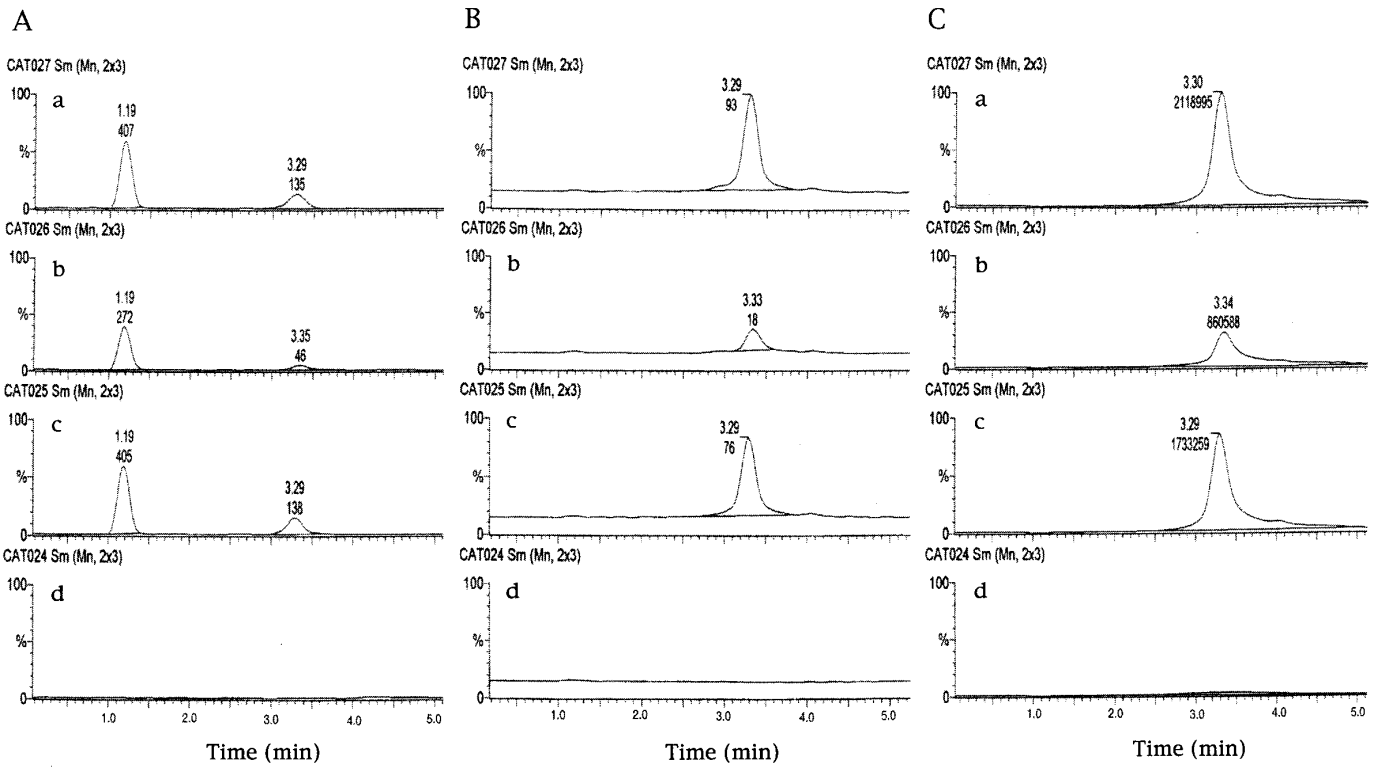

FIG. 4. Representative LC-radio-UV-MS traces for the reproducibility and in relation to the enzyme amount. The traces of the reaction without the enzyme are also shown in each bottom figure. (A) Radioactivity; (B) UV peak at 360 nm; (C) selected ion recording of MS 141.0. The numbers above each peak represent the retention time (upper) and area without decay correction (lower).

tity of this material must be established in the near future.

From these results, we consider that a useful method employing oxygen-15 has been established for the first time to study certain oxygenase reactions, although it is far from being stoichiometric. In continuing studies, we will proceed to the stoichiometry with correction for decay and the study of cellular oxygen metabolism with a lower flow of oxygen. Again, this method might be a quite good substitute for previous stable isotope methods that require a firm purification step and are only applicable to purified enzyme systems. By use of this new methodology, unknown metabolic pathways involving oxygen might be investigated; and the use of positron emitter oxygen-15 might also be especially valuable for research on oxygen chemosensors and the binding of reactive oxygen species in cellular pathology.

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