SC 11035

Peroxidase as oxidase: Reaction between $^{18}O_2$ and dihydroxyfumarate

When horse-radish peroxidase (EC 1.11.1.7) acts as oxidase upon dihydroxyfumarate, monodehydrodihydroxyfumarate and an activated form of oxygen are intermediates[1-3], but the structure of the oxygen derivative is uncertain. In the present study we have examined the reaction between oxygen and dihydroxyfumarate in the presence of peroxidase, using $^{18}O_2$ as a tracer.

Crystalline Japanese radish peroxidase was generously given by Dr. Y. MORITA. Dihydroxyfumaric acid was synthesized from tartaric acid[4] and thrice recrystallized from acetone to remove traces of cation contaminants. The preparation had ε_M (at 292 mμ) = 8890 (water), and ε_M (at 308 mμ) = 8350 (ether). Disodium dihydroxytartrate prepared by the FENTON method[5] was a gift from Dr. H. STAFFORD. Acetate buffers were prepared from redistilled glacial acetic acid and NaOH which had been passed through Dowex-50 (Na^+). Water was deionized and distilled. Oxygen containing 10 atom% ^{18}O was prepared by electrolysis of water containing 10 atom% ^{18}O (Weizmann Institute).

Glassware was treated to remove trace-metal contaminants[6]. Manometric determinations of oxygen consumption were carried out at 10° in Warburg manometers (KOH wells). Solid substrate was magnetically tipped into buffer and allowed to dissolve before enzyme was added. Tracer experiments with $^{18}O_2$ were carried out at 0–4° to minimize autoxidation of substrate and products, and to obtain maximum yields of the relatively insoluble sodium dihydroxytartrate. These reaction systems, which were 10-fold larger than the manometric experiments, consisted of 250 μmoles of dihydroxyfumaric acid, 500 μmoles of NaOH, and 1.8 mμmoles of peroxidase in a total volume of 40 ml of 0.05 M sodium acetate buffer placed in a 250-ml flask

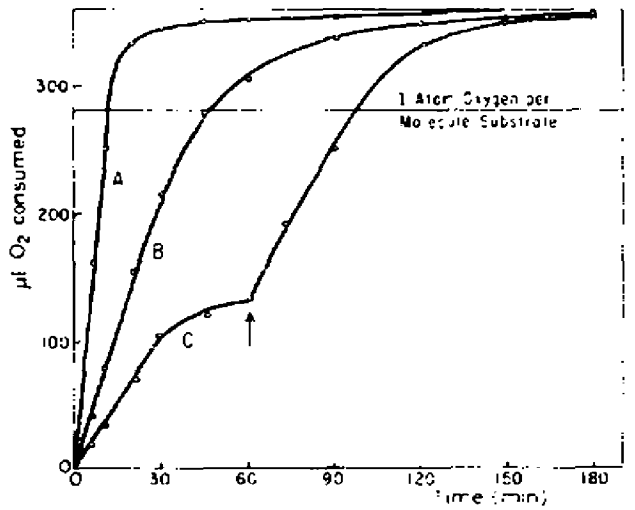


Fig. 1. Oxygen consumption by dihydroxyfumarate in the presence of varying quantities of peroxidase, at 10°. The reaction system consisted of 4.0 ml of 0.05 M sodium acetate buffer (pH 4.8) 6.25 mM in sodium dihydroxyfumarate, and (A), 450 mμM, (B) 45 mμM, and (C) 22.5 mμM peroxidase supplemented with 22.5 mμM perosidase at the arrow.

arranged for evacuation and gas exchange. Tracer experiments with $H_2{}^{18}O$ were identical, but 1.8 atom% $H_2{}^{18}O$ was used.

The ^{18}O content of labelled contents was determined by mass spectrometry of CO_2 formed after UNTERZAUCHER pyrolysis[7]. Since sodium salts are incompletely pyrolyzed due to sodium oxide formation, the samples were mixed with purified carbon (Wyex Compact Black carbon, J. M. Huber Co.). Even after heating this carbon in a stream of purified nitrogen, a small oxygen blank was obtained, and the oxygen incorporation values have been corrected, assuming that the normal oxygen blank (no pyrolyzed material), the carbon oxygen, and all the oxygen atoms of the sample contributed oxygen proportionately.

Oxygen was consumed by the peroxidase–dihydroxyfumarate system in a rapid primary phase and a slow secondary phase. In the presence of sufficient amounts of peroxidase, the primary consumption amounted to 1.1–1.2 atoms per molecule (Fig. 1). No substrate could be spectrophotometrically detected in solution at the end of the primary phase. In the presence of small amounts of enzyme, the oxygen consumption ceased prematurely, but resumed upon addition of more enzyme (Fig. 1). At 20°, oxygen consumption increased to 1.5 atoms per molecule of substrate.

In the labelling experiments, yields of 60–70% of disodium dihydroxytartrate were recovered by cooling the system to 0°, filtering the white precipitate, washing with cold water and ethanol and finally drying *in vacuo* over P_2O_5 at 80°. The infrared spectrum of the insoluble product was identical to that of an authentic sample of sodium dihydroxytartrate. The elementary analysis (Huffman Microanalytical Laboratory) was: C, 18.12, 18.04; H, 3.34, 3.23; Na, 17.72, 17.70; and O, 60.3, 58.4%. Values calculated for $Na_2C_4H_4O_8 \cdot 2^1/_2\ H_2O$ are: C, 17.72; H, 3.35; O, 61.97; and Na, 16.61%.

When the reaction was carried out in the presence of $^{18}O_2$ and H_2O, a small amount of oxygen from the atmosphere was found to be incorporated in the product (Table I). The incorporation of label from $H_2{}^{18}O$ was much greater, averaging 5.4 atoms per molecule, but the results were irregular. Dihydroxyfumarate in the absence of enzyme, but in otherwise identical conditions, incorporated 3.2 atoms

TABLE I

OXYGEN LABELLING OF DIHYDROXYTARTRATE FORMED IN THE DIHYDROXYFUMARATE–PEROXIDASE–OXYGEN REACTION

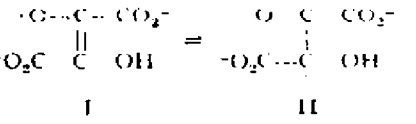
System	*Atoms ^{18}O/mol. incorporated**
$^{18}O_2 + H_2O$	0.20
	0.09
	0.33
	0.42 (0.26)
$O_2 + H_2{}^{18}O$	4.02
	6.82 (5.42)
$H_2{}^{18}O$ + dihydroxytartrate	0.76
	0.82 (0.79)
$H_2{}^{18}O$ + dihydroxyfumarate	3.54
	2.83 (3.23)

* Figures in parentheses give the average value.

per molecule. The product, dihydroxytartrate, incorporated 0.8 atom per molecule under the same conditions, but it was equilibrated as a relatively insoluble solid.

It has been reported that in the dihydroxyfumarate peroxide system, 1 atom (refs. 8, 9), 1.6 atoms (ref. 10) and 2 atoms (ref. 11) of oxygen are consumed per molecule of substrate oxidized. Our results show that oxygen consumption occurs in two phases, one rapid and the other slow (*cf.* ref. 9). Since the rapid primary phase is associated with the disappearance of substrate, the secondary phase is probably connected with the breakdown of product and the formation of tartronate, mesoxalate, and oxalate[12], but conditions leading to a primary consumption of more than 1 atom per molecule may reflect the accumulation of H_2O_2 (ref. 1).

We cannot interpret the small degree of labelling of product with oxygen from $^{18}O_2$ because the high rates of exchange of substrate and product oxygens with $H_2{}^{18}O$ mask the true degree of labelling. The observed amounts of labelling are, however, 10 times greater than could be accounted for by binding of $H_2{}^{18}O$ formed



I II

by the reduction of $^{18}O_2$ and are therefore real. Perhaps the simplest hypothesis involves the assumption that monodehydrodihydroxyfumarate exists as a resonance hybrid (I and II), capable of direct reaction with oxygen and subsequent reduction to dihydroxytartrate, but our results gave no real clue to the nature of the activated oxygen.

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Department of Biochemistry,
University of Oregon Medical School,
Portland, Oreg. (U.S.A.)

F. K. ANAN*
HOWARD S. MASON

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* Present address: Department of Biochemistry, Tokyo Medical and Dental University, Tokyo (Japan).