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DIOXYGEN ACTIVATION BY PUTIDAMONOOXIN:

SUBSTRATE-MODULATED REACTION OF ACTIVATED DIOXYGEN

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In the presence of the NADH-putidamonooxin oxidoreductase, NADH, and $^{18}O_2$, and with 4-vinylbenzoate as substrate, the monooxygenase putidamonooxin catalyzes a dioxygenase reaction producing mainly 4-glycylbenzoate. Mass spectrometry of the isolated product showed that both atoms of the $^{18}O_2$ molecule were incorporated into the substrate molecule. The rates of NADH oxidation and of O_2 uptake were between those found with benzoate and 4-methoxybenzoate, the physiological substrate of putidamonooxin.

INTRODUCTION

In the presence of the NADH-putidamonooxin oxidoreductase, NADH, and O_2, putidamonooxin, an oxygenase from Pseudomonas putida, acts as a monooxygenase on its physiological substrate, 4-methoxybenzoate. It catalyzes the O-demethylation of this substrate by oxidative attack at the substrate's methyl group (1). Besides the alkyl moiety of the substrate and of other benzoic acid derivatives, the aromatic ring is also hydroxylated by this fairly non-specific enzyme if 4-hydroxy- or 3-hydroxybenzoate is used as a substrate analogue (2). In this communication we show that putidamonooxin can be directed to a dioxygenase reaction by the use of a *substrate-induced modulation*.

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MATERIALS AND METHODS

4-Vinylbenzoate and 4-glycylbenzoate were synthesized according to published procedures (3-6). Ninety-nine-per-cent enriched $^{18}O_2$ was purchased from Amersham-Buchler, Braunschweig, F.R.G. The two components of the 4-methoxybenzoate monooxygenase were purified as described earlier (7,8). The protein contents were determined according to the method of Lowry et al. (9), with bovine serum albumin and desalted, freeze-dried putidamonooxin used as standards. NADH (grade I) and catalase (grade A, from beef liver) were obtained from Boehringer, Mannheim, F.R.G.

Enzyme assay of 4-vinylbenzoate oxygenation. The assay system contained, in 10 ml, 50 mM potassium phosphate buffer, pH 8.0, 5% ethanol, 20 mM 4-vinylbenzoate, 28 mM NADH, 0.8 mg of catalase, 2 mg of putidamonooxin, and 8 mg of reductase. The reaction was carried out at 30°C in an atmosphere of 99% enriched $^{18}O_2$ from which atmospheric oxygen was strictly excluded. After 2 h of incubation, the assay was stopped by heating the solution to the boiling point. The products were extracted into peroxide-free diethyl ether and concentrated in vacuo. The hydroxylated products were separated by thin-layer chromatography on silica gel HF 254 plates (Merck A.G., F.R.G.) developed in a solvent of benzene/methanol/propionic acid (88:8:4, v/v/v). The main product, 4-glycylbenzoate, was eluted from the silica gel with peroxide-free diethyl ether and was purified in an additional step by high-performance liquid chromatography.

High-performance liquid chromatography. The high-performance liquid-chromatography system with apparatuses from Waters and Perkin Elmer was equipped with a RP 8 column (4 x 300 mm). Elution was performed with a mixture of methanol/H_2O/H_3PO_4 (10:90:0.25, w/w/w).

Mass spectra. A Varian Type-311A mass spectrometer and a Varian 111 MS data system were used. The mass-spectra conditions were as follows: ionization energy, 90 eV; ion source temperature, 120°C; direct probe system. The product was ethylated with diazoethane prior to mass-spectral analysis.

Oxygen consumption and NADH oxidation. Oxygen consumption and the formation of hydrogen peroxide were measured polarographically with an oxygen electrode (YSI 4004, Clark-Type, built by Yellow Springs Instruments Co., Yellow Springs, Ohio, U.S.A.). NADH oxidation was followed spectrophotometrically at 366 nm.

RESULTS

To confirm our hypothesis that the iron·peroxo complex $[FeO_2]^+$ is indeed the active oxygen species of the monooxygenase putidamonooxin, we investigated whether the enzyme can also act as an external dioxygenase. Because putidamonooxin is a fairly non-specific enzyme, we looked for a substrate with which the enzyme would catalyze a dioxygenase reaction. Earlier investigations showed that benzoic acid is bound by putidamonooxin, so that the

monooxygenase exhibits an oxidase function without any substrate hydroxylation, and produces hydrogen peroxide, oxidizes NADH, and consumes O_2 in a 1:1:1 stoichiometry (2,10,11). The reason for this uncoupling reaction may be the high stability of the aromatic ring system, which does not allow the active oxygen species of putidamonooxin to form either (i) an arene oxide or (ii) a cis-dihydrodiol or (iii) a phenolic hydroxyl group directly by the insertion of an oxygen atom into a suitable C-H bond. These kinds of substances were found as products of other oxygenases attacking aromatic hydrocarbons (12-14).

Our conclusions from the experimental findings with benzoate as substrate analogue of putidamonooxin led us to look for a substance with a double bond weakened by conjugation to the ring system, in a group positioned para to the carboxyl group of benzoic acid. A suitable substrate seemed to be 4-vinylbenzoic acid. Studies on oxygen uptake, NADH oxidation, and product formation revealed that this compound is metabolized by the 4-methoxybenzoate monooxygenase with the formation of 4-glycylbenzoate. In relation to the physiological substrate 4-methoxybenzoate, the rate of NADH oxidation and oxygen uptake was 65%. The formation of hydrogen peroxide (oxidase function of the enzyme) was less than 5%. The thin-layer chromatograms of the isolated products from the enzyme assays revealed that 4-glycylbenzoate was the main product (approx. 95%) of the oxygenative 4-vinylbenzoate metabolism. Two other slight product spots could be detected on the plate but were not evaluated further. To elucidate whether the formation of 4-glycylbenzoate occurred directly by the incorporation of both atoms of the activated oxygen molecule into the substrate or by hydrolysis of an intermediate epoxide, we performed the hydroxylation under an atmosphere of 99% enriched $^{18}O_2$. The mass spectrum of the purified and ethylated

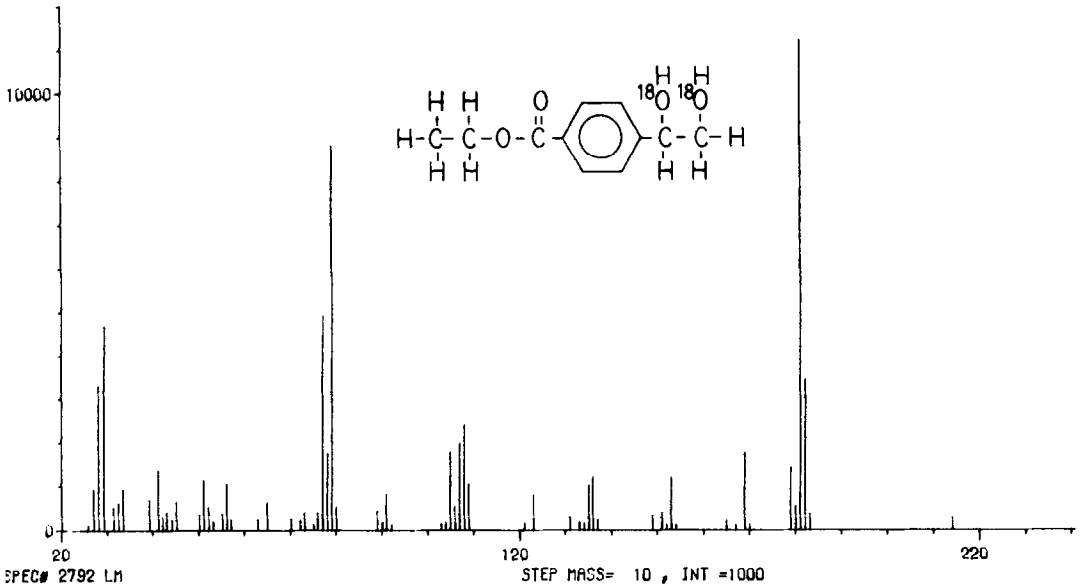

Fig. 1. Mass spectrum of the 4-[$^{18}O_2$]glycylbenzoate ethyl ester.

product is shown in Fig. 1. For the 4-glycylbenzoate ethyl ester $C_{11}H_{14}{}^{16}O_2{}^{18}O_2$ the theoretical molecular mass is 214. m/e 214 was also found from the mass spectral determination. Both the M^+ peak and the mass fragmentogram establish the structure of this compound as 4-[$^{18}O_2$]glycylbenzoate ethyl ester. The corresponding, chemically synthesized [^{16}O]-analogue ($C_{11}H_{14}{}^{16}O_4$) yielded an analogous mass spectrum with a M^+ peak at m/e 210. These results proved that both atoms of the activated oxygen molecule were indeed incorporated into the substrate molecule by putidamonooxin. The methyl ester of 4-[$^{16}O_2$]glycylbenzoate and 4-[$^{18}O_2$]glycylbenzoate yielded a M^+ peak which could not be evaluated unequivocally.

CONCLUSIONS

From our reported results in conjunction with former findings (10,11,15), we conclude that the fate of the active dioxygen species of putidamonooxin is *modulated* by the nature of the enzyme's substrate. Continuing the nomenclature introduced by

Vanneste et al. (16) (oxygen transferase for internal dioxygenase), we interpret our findings as showing that putidamonooxin with $[FeO_2]^+$ as the active oxygen complex (17-22) acts by a *substrate-modulated reaction* as either a peroxotransferase (external dioxygenase reaction [23]), or a monooxygenase, or an oxidase. The findings presented and the influence of other substrate analogues on the fate of the activated dioxygen species will be detailed in a forthcoming report.

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