Oxygenase properties of the (4-hydroxybenzoyl)methanol-cleavage enzyme from an *Alcaligenes* sp.

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Studies using H_2^{18}O and $^{18}\text{O}_2$ demonstrated that, in the cleavage of (4-hydroxybenzoyl)methanol to 4-hydroxybenzoic acid and formic acids by an enzyme from an *Alcaligenes* sp., oxygen is incorporated into both products from dioxygen and not from water. The enzyme is, therefore, an oxygenase.

INTRODUCTION

The cleavage of (4-hydroxybenzoyl)methanol into 4-hydroxybenzoic acid and formic acid is one of the steps in the catabolism of 4-hydroxyacetophenone by an *Alcaligenes* sp. (Hopper *et al.*, 1985). Extract of the organism, partially purified by ion-exchange chromatography, was used to demonstrate the reaction, which had no requirement for added cofactors but was O_2 -dependent (Hopper & Elmorsi, 1984). Although it was not clear how the O_2 was involved, the stoichiometry of its uptake approached 1 μmol of O_2 / μmol of substrate, as would be expected for a reaction catalysed by an oxygenase.

Evidence is presented here, from reactions catalysed in the presence of either H_2^{18}O or $^{18}\text{O}_2$, that the enzyme is indeed an oxygenase, incorporating an atom of oxygen from O_2 into each product.

MATERIALS AND METHODS

Preparation of enzyme

Enzyme was partially purified by ion-exchange chromatography on DEAE-cellulose as described by Hopper & Elmorsi (1984). Further purification was achieved after concentration of the pooled fractions by pressure filtration through an Amicon PM10 filter (Amicon Ltd., Stonehouse, Glos., U.K.) followed by gel-filtration on a Sephacryl S-200 column. At this stage the enzyme preparation contained only minor impurities, as indicated by polyacrylamide-gel electrophoresis or fast protein liquid chromatography on a Mono Q column (Pharmacia Fine Chemicals AB, Uppsala, Sweden). These were removed by preparative fast liquid protein chromatography on a polyanion SI-17 column. Fractions containing enzyme, from several runs, were dialysed against distilled water, freeze-dried and stored at -20°C . The purified enzyme had a specific activity of 3.4 $\mu\text{mol}/\text{min}$ per mg of protein.

Incubation with H_2^{18}O

The reaction mixtures contained 0.25 M-sodium/potassium phosphate buffer, pH 7.5, 8 mg of (4-hydroxybenzoyl)methanol and 5 mg of freeze-dried enzyme in 1 g of 98% H_2^{18}O contained in a 10 ml Erlenmeyer flask. This was incubated at 30°C for 1 h with shaking.

Incubation with $^{18}\text{O}_2$

The incubation with $^{18}\text{O}_2$ was performed in a 100 ml

Warburg-type flask the neck of which accepted the ground-glass joint of the 100 ml $^{18}\text{O}_2$ container to form a closed system. The flask contained 15 mg of (4-hydroxybenzoyl)methanol in 5 ml of 0.25 M-potassium phosphate buffer, pH 7.5, with 10 mg of freeze-dried enzyme in 0.6 ml of buffer in the side arm. It was alternately evacuated and filled with O_2 -free argon through the side-arm stopper several times and finally sealed in the evacuated state. The internal seal to the $^{18}\text{O}_2$ container was then broken, the enzyme tipped from the side arm and the flask incubated at 30°C for 1 h with shaking.

Isolation of products

Reaction mixtures were adjusted to pH 6.0 with HCl vapour and extracted twice with equal volumes of diethyl ether to remove samples of 4-hydroxybenzoic acid. The diethyl ether was evaporated off with N_2 and the dry 4-hydroxybenzoic acid purified by preparative t.l.c. on precoated silica-gel GHLF plates (Analtech, Newark, NJ, U.S.A.) in toluene/dioxan/acetic acid (90:25:4, by vol.) (Pastuska, 1961). The band corresponding to 4-hydroxybenzoic acid, when a plate was viewed under u.v. light, was scraped off and eluted with diethyl ether. The ether was evaporated off leaving a white solid that was used for mass-spectral analysis.

The aqueous reaction mixture from the $^{18}\text{O}_2$ incubation, after extraction of 4-hydroxybenzoic acid, was divided into two and the water removed from each portion on a rotary evaporator, leaving a white solid. This contained the second product of the reaction, formic acid (Scheme 1 below), as its potassium salt and each sample was reacted with derivatizing reagent to make esters of the formic acid.

The formic acid product was isolated from the H_2^{18}O reaction mixture by a slightly different procedure so that the changed H_2^{18}O could be recovered for re-use. The mixture, after extraction of 4-hydroxybenzoic acid, was acidified to pH 1 with HCl vapour and extracted three times with 1 ml of diethyl ether. The pooled diethyl ether extracts were then extracted with 2 ml of water containing sufficient K_2CO_3 to neutralize the formic acid, and the diethyl ether was evaporated with N_2 . The aqueous extract was divided into two and each portion evaporated to dryness on a rotary evaporator. The solid remaining contained the formic acid as its potassium salt.

Preparation of formic acid derivatives

Esters of formic acid were prepared by reaction of the potassium salt with 4'-bromophenacyl bromide or 4'-phenylphenacyl bromide with 18-crown-6 as catalyst as described by Grushka *et al.* (1975). The derivatizing reagent consisted of 0.1 M substituted phenacyl bromide and 5 mM-18-crown-6 in acetonitrile. To each sample was added 1 ml of reagent in a stoppered tube and the mixture incubated at 80 °C for 1 h. Each mixture was then evaporated to dryness with N₂ and the residue extracted with diethyl ether. The formate esters were purified from the diethyl ether extract by preparative t.l.c. as described for the isolation of 4-hydroxybenzoic acid, but with methylene chloride as solvent for a first separation and the product from this then purified on a second plate with toluene as solvent. The final diethyl ether extract of the silica gel was evaporated to dryness and the products examined by mass spectrometry.

Mass spectra

Mass spectra of the products were determined on a Kratos MS25 mass spectrometer with a DS55 data system by a direct-insertion-probe sampling technique. The probe temperature was 45 °C and an ionization potential of 70 eV was used.

U.v. spectra

Samples (10 µl) of reaction mixtures were added to 3 ml portions of 42 mM-KH₂PO₄/NaOH buffer, pH 7.0, and the spectra were recorded on a Pyc-Unicam SP.8-150 spectrophotometer.

Chemicals

H₂¹⁸O (98 atom% ¹⁸O) and ¹⁸O₂ (99%) were from Amersham International, Amersham, Bucks., U.K. (4-Hydroxybenzoyl)methanol was prepared by the procedure described by Robertson & Robinson (1928). 4'-Bromophenacyl bromide, 4'-phenylphenacyl bromide and 4-hydroxybenzoic acid were from BDH Chemicals, Poole, Dorset, U.K. 18-Crown-6 was from Aldrich Chemical Co, Gillingham, Dorset, U.K.

RESULTS AND DISCUSSION

In order to determine whether the oxygen atoms incorporated into the products, 4-hydroxybenzoic and formic acids, during the enzymic cleavage of (4-hydroxybenzoyl)methanol are derived from water or O₂, purified enzyme and substrate were incubated in water containing 98% ¹⁸O and exposed to normal atmospheric O₂ in one experiment, whereas in a second experiment H₂¹⁸O was used with an atmosphere of 99% ¹⁸O₂. In each experiment examination of the u.v. spectrum of the incubation mixture after 1 h showed that conversion of substrate into 4-hydroxybenzoic acid was complete. The products were isolated and, to facilitate its handling, the formic acid was esterified to either its 4'-phenylphenacyl ester or its 4'-bromophenacyl ester. Formation of these esters from the corresponding substituted phenacyl bromides ensures retention of both oxygen atoms of the formic acid in the derivative and hence total retention of any incorporated label. The formate esters and the 4-hydroxybenzoic acid were then subjected to mass-spectral analysis.

The results for 4-hydroxybenzoic acid are shown in

Fig. 1. Mass spectra of 4-hydroxybenzoic acid

The mass spectra were determined for (a) authentic 4-hydroxybenzoic acid, (b) product from the enzymic cleavage of (4-hydroxybenzoyl)methanol in H₂¹⁸O and (c) product from the enzymic cleavage of (4-hydroxybenzoyl)methanol in ¹⁸O₂.

Fig. 1. The spectrum for product from incubation with H₂¹⁸O (Fig. 1b) is very similar to that for authentic unlabelled 4-hydroxybenzoic acid (Fig. 1a), with a large peak for the molecular ion at *m/z* 138 and a major peak at *m/z* 121 due to loss of OH from the carboxy group. This indicates that most of the incorporated oxygen was derived from atmospheric ¹⁸O₂, and this suggestion is confirmed by the spectrum of the 4-hydroxybenzoic acid produced in the presence of ¹⁸O₂ (Fig. 1c). Here the molecular ion is at *m/z* 140 indicative of incorporation of an atom of oxygen from O₂. This represents over 90% incorporation, with only a small peak for unlabelled compound at *m/z* 138. The high incorporation is also shown by the near-equal intensities of the peaks at *m/z* 121 and 123, as the two oxygen atoms in the carboxy group are equivalent and either may be lost as OH. This also confirms that the label was in the carboxy group. The rest of the spectrum is identical with that for

Fig. 2. Mass spectra of (4-phenylbenzoyl)methyl formate

The mass spectra in the region of the molecular ion are shown for the 4-phenylphenacyl esters of (a) authentic formate, (b) formate produced by enzymic cleavage of (4-hydroxybenzoyl)methanol in H_2^{18}O and (c) formate produced by enzymic cleavage of (4-hydroxybenzoyl)methanol in $^{18}\text{O}_2$.

authentic unlabelled material including m/z 93 ($-\text{CO}_2\text{H}$). There was some labelling of the product by H_2^{18}O , as shown by the small peaks at m/z 140 and 123 in Fig. 1(b), but this represents only about 10% of the product. The reason for this labelling is not yet clear. It does not appear that it was due to chemical or enzyme-catalysed exchange between product and H_2^{18}O , since similar incubations of 4-hydroxybenzoate and formate with H_2^{18}O in the presence or absence of enzyme did not result in any detectable incorporation. It is, however, possible that there may have been some exchange between H_2^{18}O and the substrate, (4-hydroxybenzoyl)methanol, during the course of the incubation, but this has not been tested. This would also account for the apparent slightly higher incorporation of $^{18}\text{O}_2$ (Fig. 1c) than of ^{18}O (Fig. 1b) into 4-hydroxybenzoic acid.

A similar picture was given by the results for formic acid. The molecular ion for (4-phenylbenzoyl)methyl formate was, as expected, at m/z 240 (Fig. 2a), though at a much lower intensity than the base peak at m/z 181. The ester of formic acid produced in the presence of

Scheme 1. Incorporation of $^{18}\text{O}_2$ by (4-hydroxybenzoyl)-methanol oxygenase

H_2^{18}O showed very little labelling by ^{18}O (Fig. 2b), indicative of incorporation of ^{16}O from O_2 . This was confirmed by the increase in the molecular ion to m/z 242 for the ester of formic acid from the $^{18}\text{O}_2$ incubation mixture showing over 90% incorporation of ^{18}O with very little unlabelled ester at m/z 240 (Fig. 2c).

The mass-spectral data for the (4-bromobenzoyl)methyl formate samples gave essentially the same picture. The esters of authentic unlabelled formic acid and the product from incubation with H_2^{18}O both gave molecular ions of almost equal intensity at m/z 242 and 244 as expected for derivatives containing the two isotopes of bromine of mass 79 and 81 in their natural abundances of 50.54% and 49.56% respectively. The relative intensities were about 10% of the base peaks at m/z 183 and 185. For the ester of formate produced by incubation with $^{18}\text{O}_2$ the molecular ions were at m/z 244 and 246, indicative of incorporation of one atom of ^{18}O , with only a small peak at m/z 242, representing less than 10% of the product.

Thus oxygen from O_2 is incorporated into both products of the reaction as shown in Scheme 1 and the enzyme is, therefore, an oxygenase. It was suggested (Hopper & Elmorsi, 1984) that the reaction might be analogous to the unusual type of oxygenase activity exhibited by ribulose 1,5-bisphosphate carboxylase (EC 4.1.1.39), in which ribulose 1,5-bisphosphate is split to give glyceralate 3-phosphate and glycolate 2-phosphate, in that cleavage is between carbon atoms bearing oxo and hydroxy groups and yields two acids. However, the patterns of O_2 incorporation for the two enzymes are clearly different, as ribulose 1,5-bisphosphate carboxylase inserts oxygen from O_2 into only one of the products, the glycolate 2-phosphate, whereas that in the glyceraldehyde 3-phosphate comes from water (Lorimer *et al.*, 1983), and in this aspect (4-hydroxybenzoyl)methanol oxygenase resembles the dioxygenase type of enzyme more commonly associated with fission of the benzene ring than with modification of ring substituents. These include catechol 1,2-dioxygenase (EC 1.13.11.1) and protocatechuate 3,4-dioxygenase (EC 1.13.11.3), which cleave between adjacent carbons bearing hydroxy substituents (Hayaishi, 1962) and such a grouping would be apparent in the enol form of (4-hydroxybenzoyl)-methanol as the enediol side chain.

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