The Separation of Vanillate O-Demethylase from Protocatechuate 3,4-Oxygenase by Ultracentrifugation

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1. Protocatechuate 3,4-oxygenase in the soluble part of a cell-free extract of *Pseudomonas fluorescens* (strain T) sedimented more rapidly than vanillate O-demethylase under specified conditions in a preparative ultracentrifuge. 2. The supernatant from this process contained vanillate O-demethylase and formaldehyde dehydrogenase, and when supplemented with NADH oxidized vanillate with an uptake of 1 mole of oxygen/mole of substrate and accumulation of protocatechuate. 3. This uptake was decreased to 0.5 mole/mole of substrate in the presence of semicarbazide as trapping agent for formaldehyde. 4. Reasons are presented for the process of methyl group removal from vanillate being oxidative demethylation.

Demethylation of vanillate was investigated by Cartwright & Smith (1967) by using *Pseudomonas fluorescens* (strain T) in a study of the bacterial degradation of compounds related to lignin. A cell-free extract of ferulate-induced organisms was described that demethylated vanillate with the eventual production of β -oxoadipate from the aromatic ring and, sequentially, formaldehyde and formic acid from the methyl group. The demethylating enzyme and formaldehyde dehydrogenase remained in the supernatant fraction of ultrasonically disrupted cells after centrifugation at 72 000 $g_{\max}$ for 30 min.

Several established fractionating procedures used in attempts to purify the demethylating enzyme with a view to defining its cofactor requirements and establishing the status of protocatechuate as a metabolic intermediate led to irreversible inhibition of vanillate oxidation and, because of the lability of the enzyme system, an attempt was made to separate the demethylase by high-speed centrifugation. In periods of up to 3 hr. at 161 000 $g_{\max}$, particulate material was sedimented with concomitant increase in the specific activity of the demethylase in the supernatant; and after 6 hr. both the demethylase and protocatechuate oxygenase were sedimented.

In the work now described a fraction was obtained that, when supplemented with NADH, rapidly oxidized vanillate but showed little activity towards protocatechuate.

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METHODS

The materials and methods were similar to those used by Cartwright & Smith (1967). Phosphate buffer (67 mM) instead of saline was used for suspending cells during disruption and subsequent dilution of extracts because this resulted in more consistent enzyme activity. Other small variations are specified in the text.

Thin-layer chromatography was carried out on fluorescent silica gel (Camag D.S.F.S.; Camag, Mullenz, Switzerland) with benzene-dioxan-acetic acid (90:25:4, by vol.). The chromatograms were examined under u.v. light and by spraying with diazotized I.C.I. 5091 (4-aminophenyl 2-diethylaminoethyl sulphone).

The starting material for the fractionation described in the results was a cell-free extract (fraction F1) of *Pseudomonas* T, prepared by ultrasonic disruption in phosphate buffer, pH 7.8, followed by centrifugation at 72 000 $g_{\max}$ in the 50 angle rotor of a Beckman Spinco model L ultracentrifuge for 30 min. to remove coarse particulate matter and dilution in the same buffer to a concentration of 35 mg. of protein/ml.

RESULTS AND DISCUSSION

Centrifugation of fraction F1 for 5 hr. at 134 000 $g_{\max}$ in the 50 angle rotor of the Spinco model L ultracentrifuge gave rise to three distinct layers: (1) a pale-yellow clear supernatant; (2) an intermediary layer of micro-particulate material; (3) a pink gelatinous pellet. By using established manometric procedures, respirometric examination of the two upper layers for oxidative activity towards vanillate and protocatechuate showed that the rate of protocatechuate oxidation by the supernatant layer was rather less than half that

Fig. 1. Separation of vanillate demethylase and protocatechuate oxygenase. Each Warburg flask contained 0.2 ml. of 20% (w/v) KOH in the centre well, 2.0 μ moles of substrate and 67 mM-phosphate buffer, pH 7.8, to 1.8 ml. The incubation temperature was 30°, the gas phase air; fraction F2 equivalent to 5.0 mg. of protein was added from the side arm to initiate the reaction. NADH (1.0 μ mole) was added where indicated. The oxygen uptakes are corrected for endogenous respiration. ●, Vanillate; ○, vanillate+NADH; Δ, protocatechuate. Fraction F2 was obtained from a cell-free extract (fraction F1) of *Pseudomonas* T as described in the text.

given by the intermediate layer. Both fractions catalysed consumption of 1 mole of oxygen/mole of protocatechuate oxidized. Unsupplemented supernatant oxidized vanillate at a low rate that was increased approximately sixfold by the addition of NADH with a total uptake of 2 moles of oxygen/mole of vanillate. This gave the first indication that centrifugation might be a possible means of separating the demethylase from protocatechuate 3,4-oxygenase.

The supernatant clear layer (fraction F2) that resulted from centrifugation of fraction F1 at 164 000 g_{max} for 6 hr. in the 60 angle rotor of a Christ Omega II preparative ultracentrifuge (Martin Christ, Osterode, Harz, W. Germany) was sufficiently free from protocatechuate oxygenase to allow the production of protocatechuate from vanillate to be repeatedly and unequivocally demonstrated by chromatography. Fraction F2 catalysed low rates of oxidation of vanillate.

Table 1. Production of protocatechuate from vanillate

Each double-side-arm Warburg flask contained 0.2 ml. of 20% (w/v) KOH in the centre well, 2.0 μ moles of substrate, 1.0 μ mole of NADH and 67 mM-phosphate buffer, pH 7.8, to 1.8 ml. The incubation temperature was 30°, the gas phase air; fraction F3 equivalent to 5.8 mg. of protein was tipped from the side arm to give a total volume of 2.0 ml. After the initial rapid oxygen uptake, 0.4 ml. of fraction CF1 containing 7.2 mg. of protein was added from the second side arm. Fractions F3 and CF1 were obtained from a cell-free extract (F1) of *Pseudomonas* T by ultracentrifugation as described in the text.

Substrate	Initial O ₂ uptake in the presence of fraction F3 (mole of O ₂ /mole of substrate)	Theoretical uptake (mole of O ₂ /mole of substrate)
Vanillate	0.89	1.0
Protocatechuate	0.05	1.0
	Further uptake after adding fraction CF1 (mole of O ₂)	
Vanillate (treated with fraction F3)	0.84	1.0
Protocatechuate (treated with fraction F3)	0.76	1.0
Protocatechuate (not previously treated with fraction F3)	0.94	1.0

However, supplementation with NADH produced a 15-fold increase in activity during a rapid initial uptake corresponding to 1 mole of oxygen/mole of vanillate oxidized (Fig. 1); this value included the oxygen required for the oxidation of formaldehyde to formate by formaldehyde dehydrogenase still present in fraction F2. The initial oxidation was followed by a slower absorption comparable with that given by fraction F2 in the presence of protocatechuate alone. After 40 min. incubation, fluids from Warburg flasks containing vanillate supplemented with NADH, protocatechuate and the respective endogenous controls were deproteinized with 10% (w/v) trichloroacetic acid (0.1 vol.) and the precipitate was removed by centrifuging. The clear solutions were extracted continuously with ether for 8 hr. and, after concentration, the extracts were examined by thin-layer chromatography, when spots from vanillate incubations identical in R_F , intensity and colour with those given by authentic protocatechuate were obtained.

Separation of the demethylase under the above

conditions is apparently at variance with the earlier findings of Cartwright & Smith (1967) that centrifugation at 161 000g_{max}. completely sediments this enzyme. However, when the preparation of extracts was standardized as far as possible and protein concentration carefully adjusted, the separation was almost completely reproducible. Further trials showed that centrifugation of fraction F1 at 350 000g_{max}. for 3 hr. in the 60 angle rotor of the Christ Omega II centrifuge achieved sharper and more consistent separation of vanillate O-demethylase from protocatechuate 3,4-oxygenase. As there were no definite demarcations in the supernatant under these conditions, a fraction (F3) was arbitrarily taken as the upper 3.5 ml. of 10 ml. of material contained in the centrifuge tube, contamination by protocatechuate oxygenase increasing if a larger volume was taken. The remainder of the supernatant above the pellet was designated fraction CF1 and was used with fraction F3 to determine manometrically whether protocatechuate production from vanillate was stoicheiometric. Controls for these experiments consisted of flasks containing both fractions and protocatechuate; at zero time fraction F3 was tipped and fraction CF1 added after cessation of the rapid initial oxygen uptake. The results (Table 1) indicated stoicheiometric production of protocatechuate from vanillate.

Final confirmation of the identity of protocatechuate as the intermediate on the breakdown pathway of vanillate immediately preceding ring-fission was provided by its isolation and identification from incubation of vanillate with fraction F3. A pilot flask containing 5 μ moles of vanillate was set up in conjunction with five flasks each containing 20 μ moles of vanillate and the corresponding quantities of nucleotide and enzyme. After completion of the initial rapid oxygen consumption had been registered on the pilot flask, all flask contents were combined and deproteinized with 10% (w/v) trichloroacetic acid and centrifuged. The supernatant was continuously extracted with ether for 8 hr. Evaporation of the solvent from the extract left a solid residue, which was taken up in 2 ml. of hot water and, on cooling, slightly discoloured needles (approx. 10 mg.), m.p. 191° (undepressed on admixture with authentic protocatechuate acid), separated. The product gave a colour reaction with ferric chloride identical with that of the authentic substance.

Cartwright & Smith (1967) considered possible modes for the demethylation of vanillate and, on the evidence available then, tentatively suggested that the initial reaction was oxidative and involved the release of formaldehyde and conversion of the methoxyl group into a hydroxyl group. With the purer extracts now available, greater significance can be attached to the observed oxygen con-

sumption in the process. Although fraction F3 still contained formaldehyde dehydrogenase, as well as demethylase, action of the former enzyme was inhibited by trapping formaldehyde as its semicarbazone. Under these conditions protocatechuate accumulated during the consumption of 0.5 mole of oxygen/mole of vanillate. The rate and extent of oxygen consumption catalysed by the small amount of protocatechuate 3,4-oxygenase also present did not invalidate this result, as can be seen from Fig. 1. Under the same conditions fraction F3 converted 3-methoxybenzoate and 3,4-dimethoxybenzoate (veratrate) with the consumption of 0.5 mole of oxygen/mole of substrate into 1 mole of formaldehyde and, respectively, 3-hydroxybenzoate and 3-hydroxy-4-methoxybenzoate (isovanillate), which were not further attacked. Recovery of formaldehyde is recorded in Table 2.

Unless an undetected intermediate is formed during the production of protocatechuate and formaldehyde from vanillate, demethylation rather than demethoxylation must be the mode of methyl group removal by *Pseudomonas* T. The observed oxygen consumption in the process is incompatible with most of the other reactions suggested in Scheme 3 of Cartwright & Smith (1967). However, non-oxidative elimination of formaldehyde with the formation of 4-hydroxybenzoate and conversion into protocatechuate via a dihydrodiol would entail an identical oxygen uptake. Reasons for excluding 4-hydroxybenzoate as an intermediate were discussed in the earlier paper (Cartwright & Smith 1967). The accumulation of 3-hydroxybenzoate from 3-methoxybenzoate and lack of attack on benzoate itself, in the presence of crude and purified extracts, constitute indirect evidence against the existence in *Pseudomonas* T of enzymes catalysing

Table 2. *Formaldehyde production from substrates in the presence of fraction F3 and semicarbazide*

Substrate (2 μ moles)	Yield of formaldehyde (μ moles)	Yield (% of theoretical)
Vanillate	1.64	82.0
Veratrate	1.64	82.0
3-Methoxybenzoate	1.49	74.5
Formaldehyde (control)	1.84	92.0
Isovanillate	0	0
4-Methoxybenzoate	0	0
o-Vanillate	0	0

the hydration or hydroxylation of a precursor of protocatechuate. Hydrolytic cleavage of the ether linkage to methanol and protocatechuate is unlikely, since there is no evidence of the oxidation of methanol by whole cells, crude or partially purified extracts under a range of conditions. Work with $^{18}\text{O}_2$ and H_2^{18}O by Renson (1964) on the metabolism of 4-methoxyacetanilide to 4-hydroxyacetanilide by microsomes indicated no incorporation of ^{18}O into the demethylated product, thus suggesting that demethylation rather than demethoxylation also occurs in animal tissues.

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