STUDIES ON MECHANISM OF DOUBLE HYDROXYLATION I. EVIDENCE FOR PARTICIPATION OF NADH-CYTOCHROME c REDUCTASE IN THE REACTION OF BENZOATE 1,2-DIOXYGENASE (BENZOATE HYDROXYLASE)

Mutsuo Yamaguchi, Takashi Yamauchi, and Hitoshi Fujisawa

Department of Biochemistry, Asahikawa Medical College, Asahikawa 071-01, Japan

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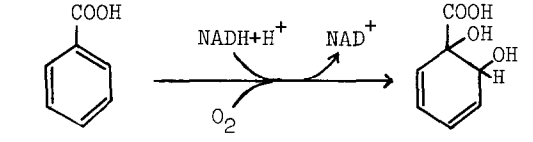
Summary. Benzoate 1,2-dioxygenase system which catalyzed double hydroxylation of benzoate was obtained from Pseudomonas arvilla and was shown to consist of two protein components (component A and B). Component A which was purified and was shown to be homogeneous upon sodium dodecyl sulfate disc gel electrophoresis retained high activity of NADH-cytochrome c reductase. Both of benzoate 1,2-dioxygenase activity and NADH-cytochrome c reductase activity were simultaneously induced by benzoate. Dichlorophenolindophenol which could serve as an electron acceptor of the NADH-cytochrome c reductase inhibited the activity of benzoate 1,2-dioxygenase. These results suggest the possibility that NADH-cytochrome c reductase activity is required for benzoate 1,2-dioxygenase.

The mechanism of single hydroxylation reactions by monooxygenases has been extensively studied in many laboratories (1). However, the study of the reaction mechanism of double hydroxylation which is catalyzed by dioxygenases requiring NADH or NADPH as an electron donor has not progressed, since these enzymes are unstable during purification and pure enzymes have not been available for use in studying the mechanism of these reactions (1,2). In the reactions of double hydroxylation, the reaction products, dihydroxy compounds, are formed by the introduction of two oxygen atoms derived from the same oxygen molecule (1-4).

Recently, Reiner et al. reported that 1,2-dihydro-1,2-dihydroxybenzoic acid (DHB) is the reaction product of benzoate 1,2-dioxygenase (benzoate hydroxylase) and that oxygen atoms of the two hydroxyl groups introduced in this reaction are derived from molecular oxygen (5,6).



Benzoic acid DHB

In the present report we demonstrate that the reaction of benzoate 1,2-di-

Abbreviations: DHB, 1,2-dihydro-1,2-dihydroxybenzoic acid; SDS, sodium dodecyl sulfate; DCIP, 2,6-dichlorophenolindophenol; DTT, dithiothreitol.

oxygenase is catalyzed by two protein components and one of them is probably identical with NADH-cytochrome c reductase.

Materials and Methods

Materials. Pseudomonas arvilla C-1 (7) grown in a medium containing sodium benzoate as an inducer was used as a source of benzoate 1,2-dioxygenase system. NADH and NAD^+ were products of Oriental Yeast Company, Japan. Cytochrome c and streptomycin sulfate were obtained from Boehringer Mannheim. DHB was prepared biologically by the use of Alcaligenes eutrophus strain B9 as descrived by Reiner and Hegeman (6). This strain was kindly donated by Dr. B. F. Johnson, Department of Bacteriology and Immunology, University of California, Berkeley.

Methods. Benzoate 1,2-dioxygenase was assayed spectrophotometrically by measuring the decrease in absorbance at 340 nm of NADH or polarographically by determining the oxygen consumption (8). The assay system contained, in a final volume of 2.0 ml, 2.0 μmoles of sodium benzoate, 0.25 μmole of NADH, 0.2 μmole of $FeSO_4$, 0.05 μmole of FAD, 170 μmoles of Tris-HCl buffer (pH 8.0), and a suitable amount of enzyme solutions. The reaction was started by adding benzoate and the initial rate was recorded at 25°.

NADH-cytochrome c reductase was assayed spectrophotometrically by measuring the reduction of ferricytochrome c by NADH at 550 nm. The assay system contained, in a final volume of 2.0 ml, 0.25 μmole of NADH, 0.08 μmole of cytochrome c, 170 μmoles of Tris-HCl buffer (pH 8.0), and a suitable amount of enzyme.

DHB dehydrogenase was assayed spectrophotometrically by measuring the increase in absorbance at 340 nm of NADH. Reaction mixture contained, in a volume of 2.0 ml, 1 μmole of DHB, 0.7 μmole of NAD^+, and a suitable amount of enzyme.

One unit of the enzyme was defined as the amount of enzyme which consumed 1 μmole of substrate per min at 25°.

Protein was determined by the method of Lowry et al. (9). All spectrophotometric measurements were carried out with a Hitachi 356 recording spectrophotometer. Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis was carried out as descrived by Laemmli (10).

Results

Separation of benzoate 1,2-dioxygenase system into two components, A and B. Pseudomonas arvilla was grown at 24° with vigorous aeration in a medium containing benzoate as an inducer and a major carbon source. The medium contained, in a volume of 1 liter, sodium benzoate, 3.0 g; yeast extract, 0.5 g; $(NH_4)_2HPO_4$, 3.0 g; KH_2PO_4, 1.2 g; NaCl, 1.6 g; $FeSO_4 \cdot 7H_2O$, 0.1 g; and $MgSO_4 \cdot 7H_2O$, 0.2 g. Cells were harvested when growth reached the stationary phase and were stored frozen with little loss of activity for several months. All subsequent extraction and purification procedures were carried out at about 4° and all subsequent centrifugation was carried out at about 10,000 X g for 10 min.

The frozen cells (190 g) were suspended in 380 ml of 0.1 M Tris-HCl buffer (pH 8.0) containing 0.5 mM dithiothreitol (DTT) and were subjected to the action of a sonic oscillator for 12 min, followed by centrifugation. To the supernatant fluid (crude extract) were added 61 ml of 10 % streptomycin sulfate solution and the resulting precipitate was removed by centrifugation. The supernatant solution was brought to 40 % saturation with solid ammonium sulfate and the resulting precipitate was removed by centrifugation. The supernatant was then

Table I. Requirement of two components for benzoate 1,2-dioxygenase activity

Component added	Benzoate 1,2-dioxygenase activity (units)
A	0
B	0
A + B	0.11

Benzoate 1,2-dioxygenase activity was determined spectrophotometrically. Preparation of component A used was the 40-50 % ammonium sulfate fraction (2.9 mg protein). Preparation of component B used was the 60-70 % ammonium sulfate fraction (3.4 mg protein).

Table II. Copurification of cytochrome c reductase and component A of benzoate 1,2-dioxygenase system.

Fraction	Protein (mg)	Specific activity(units/mg) (a) Cyt. c reductase	(b) Benzoate 1,2-dioxygenase	a/b	Yield (%)
Crude extract	22,970	1.43	---*		100
Streptomycin	22,720	1.52	---*		96
Ammonium sulfate (40-50 %)	6,685	3.00	0.019	158	61
Acid treatment	1,844	9.36	0.059	159	52
Sephadex G-200	179	93.2	0.59	158	43
DEAE-Sephadex	19	496	3.16	157	29
Sephadex G-100	7.2	911	5.77	158	20

* Activities of component A could not be determined because these preparations contained component B activity.

Assay of component A was carried out by measuring the activity of benzoate 1,2-dioxygenase in the presence of component B (60-70 % ammonium sulfate fraction, 2.0 mg). It was not possible to achieve levels of the 60-70 % ammonium sulfate fraction which are nonlimiting in the assay of component A. Consequently, the assay of component A was emperical. Under the assay conditions the activity of benzoate 1,2-dioxygenase activity was proportional to the amount of component A added. The yield was calculated from the activity of cytochrome c reductase.

brought to 50 % saturation with solid ammonium sulfate and the resulting 40-50 % $(NH_4)_2SO_4$ precipitate (component A) was collected by centrifugation. The precipitate was dissolved in 200 ml of 0.1 M Tris-HCl buffer (pH 8.0) containing 0.5 mM DTT. The supernatant solution was brought to 60 % saturation with solid ammonium sulfate and the precipitate was removed by centrifugation. The supernatant was

then brought to 70 % saturation with solid ammonium sulfate and the resulting 60-70 % $(NH_4)_2SO_4$ precipitate (component B) was collected by centrifugation.

Neither component A nor B alone had significant benzoate 1,2-dioxygenase activity and both of them were required for the activity of the enzyme as shown in Table I. These components had no activity of DHB dehydrogenase which catalyzes the conversion from DHB to catechol. The activity of DHB dehydrogenase was removed by the first ammonium sulfate precipitation.

Purification of component A of benzoate 1,2-dioxygenase system. The pH of the 40-50 % $(NH_4)_2SO_4$ fraction was adjusted to 4.8 by the addition of 1 M acetic acid and the resulting precipitate was removed by centrifugation. The supernatant solution was neutralized by the addition of 1 M Tris-HCl buffer (pH 8.0) and then brought to 50 % saturation with solid ammonium sulfate. The precipitate was collected by centrifugation and dissolved in 7 ml of 0.1 M Tris-HCl buffer (pH 8.0) containing 0.5 mM DTT. The solution was chromatographed on a Sephadex G-200 column (1.9 X 106 cm) which had been equilibrated with 0.1 M Tris-HCl buffer (pH 8.0) containing 0.5 mM DTT. Active fractions which should be eluted from the column after 194 ml, in a total volume of 60 ml, were pooled and adjusted to 50 % saturation with solid ammonium sulfate. The precipitate was collected by centrifugation and dissolved in 2.0 ml of 0.1 M Tris-HCl buffer (pH 8.0) containing 0.5 mM DTT. The active solution was dialyzed for 3 hours against 0.1 M Tris-HCl buffer (pH 8.0) containing 0.1 M NaCl and 0.5 mM DTT prior to application to a DEAE-Sephadex column (1.2 X 16 cm) which had been equilibrated with the same buffer. Elution was performed with a linear gradient (total volume, 60 ml) of 0.1 M to 0.6 M NaCl in 0.1 M Tris-HCl buffer (pH 8.0) containing 0.5 mM DTT. The active enzyme which usually emerged after 35 ml, in a total volume of 12 ml of effluent, was pooled and precipitated by the addition of solid ammonium sulfate (50 % saturation). The precipitate was dissolved in 1 ml of 0.1 M Tris-HCl buffer (pH 8.0) containing 0.5 mM DTT and then chromatographed on a Sephadex G-100 column (1.2 X 106 cm) which had been equilibrated with the same buffer. The active fraction which usually emerged after 80 ml of effluent, in a total volume of 14 ml, was pooled and collected by the addition of solid ammonium sulfate (50 % saturation) The resulting precipitate was dissolved in 0.4 ml of 0.1 M Tris-HCl buffer (pH 8.0) containing 0.5 mM DTT.

The purification procedure of component A of benzoate 1,2-dioxygenase system is summarized in Table II. The purified enzyme, which was shown to be homogeneous by SDS-polyacrylamide gel electrophoresis as shown in Fig. I, retained high activity of NADH-cytochrome c reductase. As shown in Table II, the ratio of activities of NADH-cytochrome c reductase and benzoate 1,2-dioxygenase did not vary throughout the purification procedure.

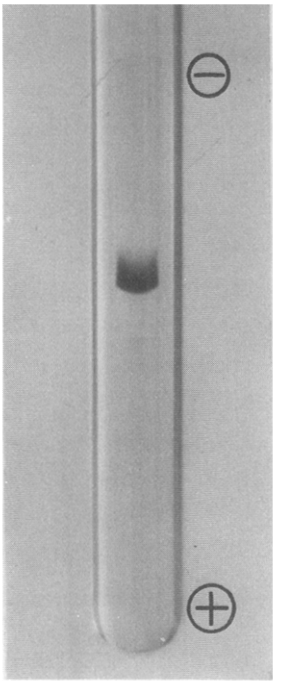


Fig. 1. SDS-polyacrylamide gel electrophoresis of the purified component A. The enzyme (60 μg of protein) was treated with SDS and 2-mercaptoethanol and submitted to electrophoresis with a 7.5 % separating gel. Migration was from top to bottom. The gels were stained with Amino black 10B.

Coinduction of benzoate 1,2-dioxygenase activity and NADH-cytochrome c reductase by benzoate. It is well known that benzoate 1,2-dioxygenase activity is induced by growth with benzoate in Pseudomonads. When it was used as growth substrate of Pseudomonas arvilla, the specific activity of NADH-cytochrome c reductase in the crude extract increased 50- to 70-fold as shown in Table III. Both of component A and B of benzoate 1,2-dioxygenase system also induced by growth with benzoate, indicating that both of them participate specifically in the metabolism of benzoate in this organism.

Inhibition of benzoate 1,2-dioxygenase activity by an electron acceptor. Purified component A of benzoate 1,2-dioxygenase system catalyzed the reduction of various electron acceptor as well as cytochrome c by NADH. The activity towards 2,6-dichlorophenolindophenol (DCIP) was approximately 10 % of the activity towards cytochrome c. In order to investigate the relationship between reductase activity and benzoate 1,2-dioxygenase activity, the effect of DCIP upon the benzoate 1,2-dioxygenase activity was studied (Fig. 2). When DCIP was added to the reaction mixture during the reaction of benzoate 1,2-dioxygenase, benzoate-dependent oxy-

Table III. Coinduction of benzoate 1,2-dioxygenase and NADH-cytochrome c reductase activity.

Carbon source added	Cyt. c reductase (units/mg)	Benzoate 1,2-dioxygenase Component A (units/mg)	Component B (units/mg)
Glucose	0.03	0	0
Succinate	0.02	0	0.001
Benzoate	1.43	0.025	0.021

Pseudomonas arvilla was grown on media containing the carbon source indicated and cells were harvested at late logarithmic phase. Activity of NADH-cytochrome c reductase was determined with the crude extract. Activities of two components of benzoate 1,2-dioxygenase system were determined after separation of two components by ammonium sulfate fractionation. Component A and B were the 40-50 % ammonium sulfate fraction and 60-70 % ammonium sulfate fraction, respectively. Activity of component A was determined in the presence of component B (60-70 % ammonium sulfate fraction, 3.4 mg) obtained from the cells grown on the benzoate medium. Activity of component B was determined in the presence of component A (40-50 % ammonium sulfate fraction, 2.9 mg) obtained from the cells grown on the benzoate medium.

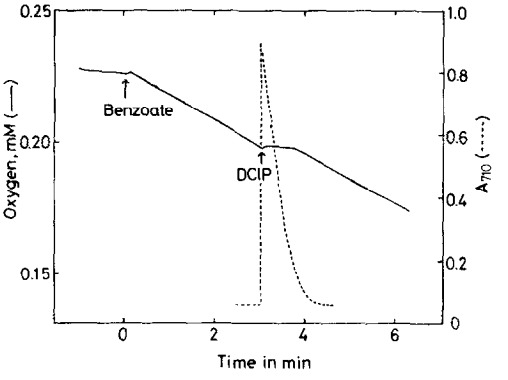


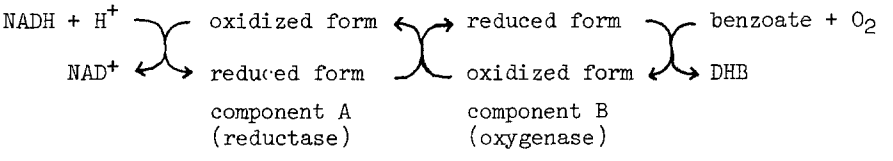
Fig. 2. Inhibition of benzoate 1,2-dioxygenase reaction by DCIP. The reaction mixture contained 1 μmole of NADH, 0.2 μmole of $FeSO_4$, 0.05 μmole of FAD, 2 μmoles of benzoate, 6.7 μg of purified component A, and 960 μg of component B (60-70 % ammonium sulfate fraction) in a total volume of 2.0 ml. Benzoate 1,2-dioxygenase activity was measured polarographically. The reaction was started by the addition of benzoate. DCIP was added at the time as indicated by the arrow at the final concentration of 0.4 mM. Reduction of DCIP by the enzyme was monitored spectrophotometrically by measuring the absorbance at 710 nm in parallel experiments under identical experimental conditions.

gen consumption was significantly inhibited. After the reduction of DCIP, which was monitored spectrophotometrically by measuring the absorbance at 710 nm, was completed, benzoate-dependent oxygen consumption was completely recovered.

Discussion

Double hydroxylation reaction was first demonstrated by Kobayashi et al. with anthranilate hydroxylase which catalyzes the formation of catechol from anthranilic acid (3). During the conversion of one mole of anthranilic acid to catechol, one mole each of oxygen and NADH is used, and one mole each of CO_2 and ammonia is produced. They demonstrated by experiments with ^{18}O that both atoms of oxygen in catechol are exclusively derived from molecular oxygen. Benzene hydroxylase was also shown to catalyze the incorporation of 1 mole of molecular oxygen into benzene (4). Both of them were shown to be resolved into two protein components (11, 12).

We resolved benzoate 1,2-dioxygenase (benzoate hydroxylase) into two components, one of which was obtained in pure form (component A) and both of which were required for benzoate 1,2-dioxygenase activity. Component A which was homogeneous upon SDS-polyacrylamide gel electrophoresis retained high activity of NADH-cytochrome c reductase and the ratio of activities of NADH-cytochrome c reductase and benzoate 1,2-dioxygenase was constant during purification, indicating that component A of benzoate 1,2-dioxygenase system was identical with NADH-cytochrome c reductase. Coinducibility of these activities by benzoate also supported this possibility. In order to clarify the possibility of the involvement of the NADH-dependent reductase activity in benzoate 1,2-dioxygenase reaction, the effect of DCIP, a poor electron acceptor of the NADH-dependent reductase, upon the benzoate 1,2-dioxygenase activity was investigated, since the velocity of the reduction of cytochrome c by NADH was too fast to be analyzed. The finding that benzoate 1,2-dioxygenase activity was inhibited by the addition of DCIP and was recovered after the completion of the reduction of DCIP by NADH suggests that the NADH-dependent reductase activity may participate in benzoate 1,2-dioxygenase reaction. These observations and similarities to monooxygenase systems taken together suggest that component A of benzoate 1,2-dioxygenase system functions as the NADH-benzoate 1,2-dioxygenase reductase as follows.



Recently, Axcell and Geary reported that benzene-oxidizing system(benzene hydroxylase) obtained from a species of Pseudomonas consisted of three protein components and they speculated the involvement of NADH-dependent reductase in benzene-oxidizing reaction (13).

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