**Subunit Structure of Oxygenase Component in Benzoate-1,2-dioxygenase System from *Pseudomonas arvilla* C-1**

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Benzoate-1,2-dioxygenase system from *Pseudomonas arvilla* C-1 consists of two protein components, benzoate-1,2-dioxygenase reductase and benzoate-1,2-dioxygenase (Yamaguchi, M., and Fujisawa, H. (1980) *J. Biol. Chem.* 255, 5058-5063). Benzoate-1,2-dioxygenase exhibited two protein bands (α and β) on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and their molecular weights were estimated to be 50,000 and 20,000, respectively. The intensities of protein staining on polyacrylamide gels suggested that these two subunits were present in equimolar quantities in benzoate-1,2-dioxygenase. Molecular weight of benzoate-1,2-dioxygenase was estimated to be 201,000 by sedimentation equilibrium (Yphantis method). The values of molecular weights of native enzyme and its subunits suggested that the subunit structure of benzoate-1,2-dioxygenase may be αβ₂. Cross-linking experiments also suggested the same subunit structure. These two subunits were separated from each other by Ultrigel AcaA44 chromatography in the presence of 6 M urea. Amino acid compositions of the two subunits were examined and compared with that of native enzyme. NH₂-terminal amino acids of α and β subunits were both serine, and isoelectric points of α and β in the presence of 6 M urea were determined to be pH 5.6 and pH 4.8, respectively. The enzyme contained 8.2 mol of iron and 5.9 mol of labile sulfide/mol of enzyme, suggesting the presence of additional iron atoms besides iron-sulfur clusters. The isolated β subunit did not contain any significant amounts of iron and labile sulfide, but the α subunit contained approximately 2 mol each of iron and labile sulfide and exhibited an absorption spectrum of binuclear iron cluster type.

**EXPERIMENTAL PROCEDURES**

**Materials—**NADH, dihtiothreitol, dansyl chloride, dansyl amino acids, DTBP (8), and cross-linked bovine serum albumin were obtained from Sigma. Amido black 10B, Coomassie brilliant blue (R-250), Coomassie brilliant blue (G-250), 2-mercaptoethanol, and urea were obtained from Nakarai Chemical Co., Kyoto, Japan. Polyamide layer sheets (9) were obtained from Cheng-Chin Trading Co., Taipei, Taiwan. Ampholine and Ultrogel AcA44 were obtained from LKB. Sephadex G-200 was obtained from Pharmacia. Oxygenase component in benzoate-1,2-dioxygenase system from *P. arvilla* C-1 (ATCC 23974) was purified as reported previously (7).

**Sedimentation Experiments—**Ultracentrifuge measurements were carried out in a Hitachi 282 analytical ultracentrifuge. Prior to sedimentation analysis, the sample was applied to a column of Sephadex G-200 equilibrated with 50 mM Tris/HCl buffer, pH 6.8, containing 5% dimethyl sulfoxide, 0.1 M NaCl, and 1 mM dihtiothreitol, and eluted with the same buffer to remove the aggregated forms of the enzyme. Sedimentation equilibrium was carried out according to the method of Yphantis (10) in a three-channel centerpiece. After centrifugation at 12,000-13,000 rpm at 4°C for 24 h, scanning was done at 280 nm by ultraviolet optics.

**Cross-linking Experiments—**Cross-linking of benzoate-1,2-dioxygenase with DTBP was carried out in 0.2 M triethanolamine/HCl buffer, pH 8.5 (11), at room temperature in a final volume of 100 μl. The reaction was quenched by the addition of 5 μl of 1 M ammonium acetate solution (8). After incubation for 10 min, 4 μmol of N-ethylmaleimide (in 5 μl of ethanol) were added to prevent disulfide-sulfhydryl exchange. For electrophoresis, 400 μl of 0.1 M sodium borate buffer, pH 8.5, containing 2% SDS, 10% glycerol, and 0.001% bromphenol blue, were added to the reaction mixture, and the mixture was heated for 3 min at 100°C.

**Disc Gel Electrophoresis—**For determination of molecular weights of benzoate-1,2-dioxygenase subunits, SDS-polyacrylamide gel electrophoresis was performed at 6 mA/gel for 4 h according to the method of Weber and Osborn (12). The gels were stained with Amido black 10B or Coomassie brilliant blue (R-250) and then scanned at 580 nm by ISCO UA-5 absorbance monitor.

For analysis of cross-linked enzyme, electrophoresis was performed at 8 mA/gel for 2 h on 5% polyacrylamide gels according to the procedure of Davies and Stark (11). Gels were stained with Coomassie brilliant blue (R-250).

**Two-dimensional Gel Electrophoresis—**Electrophoresis of the first dimension was performed on 5% polyacrylamide gels in the presence of 0.1% SDS in glass tubing (12 X 0.25 cm) essentially according to the procedure of Davies and Stark (11). After electrophoresis at 2 mA/gel for 3 h, the cylindrical gel was removed from the glass tube and placed in the slot on top of a discontinuous slab gel system similar to that of Laemmli (13). It consisted of a 3% polyacrylamide stacking gel on a 10% polyacrylamide separating gel (10 X 15 X 0.1 cm). Agarose (1%) containing 62.5 mM Tris/HCl buffer, pH 6.8, 0.1% SDS, 10% 2-mercaptoethanol, and 10% glycerol at 80°C was added on top of the stacking gel to cover the cylindrical gel. The slab gel was run at 21 mA for 3 h. An electrode buffer consisting of 0.05

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Subunit Structure of Benzoate-1,2-dioxygenase

**RESULTS**

**Molecular Weights**—Purified benzoate-1,2-dioxygenase, which was homogeneous on polyacrylamide disc gel electrophoresis (7), showed two protein bands on gel electrophoresis in 0.1% SDS as shown in Fig. 1A. The molecular weights of the two protein bands were determined to be 50,000 (designated α subunit) and 20,000 (β subunit) according to the method of Weber and Osborn (12) as shown in Fig. 2. When the two protein bands on SDS gel were stained with Amido black 10B or Coomassie brilliant blue (R-250) and scanned with a densitometer at 580 nm to determine the relative amount of protein in each band, a ratio of protein staining of 5:2 for the bands of α subunit and β subunit was obtained, indicating a molar ratio of 1:1 for α subunit and β subunit based on their molecular weights of 50,000 and 20,000.

The molecular weight of purified benzoate-1,2-dioxygenase, as determined by low speed sedimentation equilibrium, was reported to be 273,000 (7). However, the purified enzyme was found to have a tendency to aggregate as shown in Fig. 3. When the purified enzyme was stored in a frozen state at –20 °C for 1 month at a concentration of 54 mg/ml, approximately 20% of the enzyme appeared to become aggregated forms. Since the elution position of the aggregates of the enzyme preceded that of β-galactosidase, the molecular weight of the aggregates appeared to be higher than 520,000. In order to obtain an accurate value of the molecular weight of the enzyme, high speed sedimentation equilibrium analysis (10) of the enzyme was performed immediately after removal of the aggregates by gel filtration. The value, 201,200 ± 11,500 (Table 1), was considerably lower than that reported previously.

**Cross-linking Studies**—When benzoate-1,2-dioxygenase was treated with DTBP and analyzed by SDS-polyacrylamide gel electrophoresis, the patterns of protein-staining bands, as shown in Fig. 4, were obtained. The molecular weight of each band was estimated to be 43,000, 70,000, 88,000, 120,000, 145,000, 170,000, and 190,000, using cross-linked bovine serum albumin as a standard marker. These values of the molecular weights tentatively suggested that each of the bands might correspond to βα, αβ, αα, ββ, and αβ, respectively.

When the tube gel which had been electrophoresed for separation of the enzyme cross-linked with DTBP as described above was subjected to electrophoresis of the second dimension as described under “Experimental Procedures,” the patterns of protein bands as shown in Fig. 5 were obtained. All

**Fig. 1. SDS-polyacrylamide gel electrophoresis of benzoate-1,2-dioxygenase and its subunits.** Electrophoresis was carried out as described under “Experimental Procedures.” Approximately 50 μg of benzoate-1,2-dioxygenase (A), 25 μg of α subunit (B), and 15 μg of β subunit (C) were applied to each gel. After electrophoresis, the gels were stained with Amido black 10B.

**Fig. 2. Determination of molecular weights of benzoate-1,2-dioxygenase subunits by SDS-polyacrylamide gel electrophoresis.** Electrophoresis was carried out as described under “Experimental Procedures.” For calibration of the gel, the following standard proteins were used: 1, bovine serum albumin (M, 66,000); 2, catalase (M, 60,000); 3, ovalbumin (M, 43,000); 4, alcohol dehydrogenase (M, 37,500); 5, chymotrypsinogen (M, 25,700); 6, myoglobin (M, 17,200); 7, cytochrome c (M, 11,700). The arrows indicate the positions of two subunits of benzoate-1,2-dioxygenase.
cross-linked protein species were converted to the monomeric forms by the action of 2-mercaptoethanol contained in 1% agarose layer before electrophoresis of the second dimension. The species assumed to be $\alpha$, $\beta$, $\alpha\beta$, and $\alpha\beta_2$, respectively, and each subunit structure of benzoate-1,2-dioxygenase might be $\alpha\beta$.

Separation of Subunits—Benzoate-1,2-dioxygenase was separated into $\alpha$ and $\beta$ subunits by gel filtration chromatography on Ultrogel AcA44 in 50 mM Tris/HCl buffer, pH 6.8, containing 2 mM dithiothreitol, 6 M urea, and 5% dimethyl sulfoxide as shown in Fig. 6. Each subunit, $\alpha$ and $\beta$, was eluted in fractions of 38 to 46 and 48 to 58, respectively, and each

![Fig. 3. Sephadex G-200 column chromatography on the preparation of benzoate-1,2-dioxygenase stored under freezing conditions. The benzoate-1,2-dioxygenase preparation (5.4 mg) which was stored at -20 °C for 1 month at the protein concentration of 54 mg/ml in 50 mM Tris/HCl buffer, pH 6.8, containing 5% dimethyl sulfoxide, 0.1 M NaCl, and 1 mM dithiothreitol, was applied to a column (1.9 × 50 cm) of Sephadex G-200 equilibrated with the same buffer. The column was eluted in fractions of 1 ml, and the absorbance at 280 nm of each fraction was measured. For calibration of the void volume of the column, the preparation of the void volume of the column.](image-url)

![Fig. 4. SDS-polyacrylamide gel electrophoresis of benzoate-1,2-dioxygenase cross-linked with DTBP. Benzoate-1,2-dioxygenase (2 mg/ml) was cross-linked with DTBP of 0.5 mg/ml (B), 1 mg/ml (C), and 5 mg/ml (D) in 0.2 M triethanolamine/HCl buffer, pH 8.5, at room temperature for 2 h. Control experiment (A) was without treatment with DTBP. Electrophoresis was carried out as described under "Experimental Procedures." Approximately 20 $\mu$g of protein were applied to each gel. Molecular weights of protein bands were estimated using cross-linked bovine serum albumin as a standard. Based on the values of their molecular weights, the cross-linked species were tentatively supposed to be $\beta$, $\alpha\beta$, $\alpha\beta_2$, $\alpha\beta_3$, $\alpha\beta_6$, and $\alpha\beta_8$, respectively.](image-url)

![Fig. 5. Two-dimensional polyacrylamide gel electrophoresis of benzoate-1,2-dioxygenase cross-linked with DTBP. Benzoate-1,2-dioxygenase (2 mg/ml) was cross-linked with DTBP (5 mg/ml) in 0.2 M triethanolamine/HCl buffer, pH 8.5, at room temperature for 2 h. Two samples of the cross-linked enzyme, each containing 20 $\mu$g of protein, were subjected to SDS-polyacrylamide disc gel electrophoresis, and one of the two gels was stained with Coomassie brilliant blue (R-250) (A). The unstained disc gel was placed over a slab gel, and electrophoresis was carried out as described under "Experimental Procedures." After electrophoresis of the second dimension, the gel was stained with Coomassie brilliant blue (R-250) (B).](image-url)
Subunit Structure of Benzoate-1,2-dioxygenase

Absorbance at 415 nm, presumably due to iron-sulfur cluster, was eluted at a region corresponding to a subunit on Ultrogel AcA44 gel filtration. Iron determination of each fraction of the gel filtration revealed that about 70% of iron was eluted at a region corresponding to a subunit, and the remainder was eluted in the column volume. These results, taken together, indicated that an iron-sulfur cluster might lie on a subunit of benzoate-1,2-dioxygenase.

Both subunit preparations thus obtained were dialyzed against 50 mM Tris/HCl buffer, pH 6.8, containing 5% dimethyl sulfoxide and 1 mM dithiothreitol, and then used for studies described below.

Amino Acid Compositions of Subunits—The results of amino acid analyses of α and β subunits are summarized in Table II. The numbers of α and β subunits in benzoate-1,2-dioxygenase, m and n, were calculated from the results of their amino acid analyses according to the method of least squares by the following formula.

$$P = \sum_{i=1}^{18} [N_i - (ma_i + nb_i)]^2$$

where $N_i$ is the number of each amino acid residue in the native enzyme, $a_i$ is that of α subunit, and $b_i$ is that of β subunit. The following two equations must be satisfied in order to minimize $P$.

$$\frac{\partial P}{\partial m} = 2 \sum_{i=1}^{18} [N_i - (ma_i + nb_i)](-a_i) = 0$$

$$\frac{\partial P}{\partial n} = 2 \sum_{i=1}^{18} [N_i - (ma_i + nb_i)](-b_i) = 0$$

The values of $m$ and $n$ were calculated to be 2.9 and 2.8, respectively, from the two equations, supporting the contention that the subunit structure of benzoate-1,2-dioxygenase is αβ2.

NH$_2$-terminal Amino Acid Residues of Subunits—The NH$_2$-terminal amino acids of both subunits of benzoate-1,2-dioxygenase were determined according to the dansylation method as described under "Experimental Procedures." In each case, a major dansyl-serine was identified after acid hydrolysis, indicating that the NH$_2$-termini of both α and β subunits might be serine.

Isoelectric Points of Subunits—Isoelectric focusing of each subunit of benzoate-1,2-dioxygenase on polyacrylamide gel in the presence of 6 M urea revealed a single protein band with an isoelectric point of pH 5.6 for α subunit and pH 4.8 for β subunit. An isoelectric point for native enzyme was reported to be pH 4.5 (7).

Absorption Spectra of Subunits—Fig. 7 shows the visible absorption spectra of benzoate-1,2-dioxygenase and its α subunit. Both measurements were performed at the same concentration of a subunit. The native enzyme exhibited a broad absorption spectrum with maxima at about 325 and 464 nm and with a shoulder at about 560 nm; α subunit also exhibited a broad absorption spectrum with maxima at about 325, 415, and 450 nm, presumably due to iron-sulfur cluster of [2Fe-2S] type. Thus, both absorption spectra of the native enzyme and

![Absorption spectra of benzoate-1,2-dioxygenase and its α subunit. The concentration of benzoate-1,2-dioxygenase (---) was 4.2 μM and that of α subunit (-----) was 12.5 μM in 50 mM Tris/HCl buffer, pH 6.8, containing 5% dimethyl sulfoxide and 1 mM dithiothreitol.](image)
a subunit appeared to resemble each other both in shape and intensity, suggesting that the visible absorption of benzene-1,2-dioxygenase might be primarily derived from iron-sulfur cluster on a subunit. In contrast to \( \alpha \) subunit, \( \beta \) subunit showed no significant absorption in the visible range.

**Iron and Labile Sulfide Contents of Subunits**—Iron and labile sulfide contents of benzene-1,2-dioxygenase and its \( \alpha \) and \( \beta \) subunits are summarized in Table III. The iron and labile sulfide contents of the native enzyme were calculated to be 8.2 and 5.9 mol/mol of enzyme, based on a molecular weight of 201,000 of the enzyme. The labile sulfide content of \( \alpha \) subunit, 1.9 mol/mol, accounted for the total labile sulfide content of the enzyme, based on the finding that the subunit structure of the enzyme is \( \alpha \beta \). The value of the iron content of \( \alpha \) subunit, 1.8 mol/mol, corresponded to approximately 70% of the total iron content of the enzyme. These results provided the evidence for the contention that an iron-sulfur cluster of [2Fe-2S] type might be on each \( \alpha \) subunit of benzene-1,2-dioxygenase and, furthermore, suggested that additional iron atoms might be contained in the enzyme. The preparation of \( \beta \) subunit had no significant amounts of both iron and labile sulfide.

**Table III**

| Sample   | Iron   | Labile sulfide |
|----------|--------|----------------|
| Native enzyme | 8.2    | 5.9            |
| \( \alpha \) | 1.8    | 1.9            |
| \( \beta \) | 0.1    | 0.1            |

The bazoate-1,2-dioxygenase system which catalyzes the double hydroxylation of benzoate consists of two protein components, benzoate-1,2-dioxygenase reductase and benzene-1,2-dioxygenase (3-7). The former is an iron-sulfur flavoprotein containing one FAD and one iron-sulfur cluster of [2Fe-2S] type (5, 6), and the latter is an iron-sulfur protein with iron-sulfur clusters of [2Fe-2S] type (7). In the present study, this iron-sulfur protein, benzene-1,2-dioxygenase, was shown to be composed of nonidentical subunits which include a larger iron-sulfur cluster-containing polypeptide (\( \alpha \)) with a molecular weight of 50,000 and a smaller polypeptide (\( \beta \)) with a molecular weight of 20,000.

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