Nonidentical Subunits of Protocatechuate 3,4-Dioxygenase†

Ryotaro Yoshida,* Kazuko Hori,‡ Motokazu Fujiwara, Yukikazu Saeki, Hiroyuki Kagamiyama,§ and Mitsuhiro Nozaki§

ABSTRACT: Protocatechuate 3,4-dioxygenase (EC 1.13.11.3) has been reported to have a molecular weight of 700 000 and to consist of eight identical subunits, each containing one atom of ferric iron and a substrate binding site. This subunit has now been found to dissociate further into four smaller subunits of two nonidentical types ($\alpha_2\beta_2$), upon sodium dodecyl sulfate gel electrophoresis. The molecular weights of the α and β subunits were estimated to be 22 500 and 25 000, respectively. Isoelectric focusing of the enzyme in 6 M urea revealed that the isoelectric points of the α and β subunits were 5.2 and 9.5, respectively. Separation of the two subunits was achieved by chromatography on sulfopropyl (SP)-Sephadex in 6 M urea after treatment of the enzyme with 8 M urea at 37 °C for 6 h. The NH_2-terminal sequence of the α subunit was determined to be Pro-Ile-Glu-Leu-Leu-Pro-Glu-Thr-Pro-Ser-Glx-Thr-Ala-Gly and that of the β subunit, Pro-Ala-Gln-Asp-Asn-Ala-Arg-Phe-Val-Ile-Arg-Asx-Arg-Asx. Phenylalanine was found as the COOH-terminal residue of the α subunit. However, the COOH terminus of the β subunit was not detected by any of three methods employed.

Protocatechuate 3,4-dioxygenase (protocatechuate:oxygen 3,4-oxidoreductase, EC 1.13.11.3) catalyzes the cleavage of the benzene ring of protocatechuic acid between the two hydroxylated carbon atoms with insertion of two atoms of molecular oxygen to form β-carboxymuconic acid (Stanier and Ingraham, 1954). The enzyme was obtained in a crystalline form from *Pseudomonas aeruginosa* and shown to contain eight atoms of ferric iron per mole of enzyme, based on the molecular weight of 700 000 (Fujisawa and Hayaishi, 1968). The fact that the enzyme is dissociated into homogeneous subunits of approximately 90 000 daltons by an alkaline treatment, together with an electron microscopic observation, has led us to conclude that the enzyme consists of eight identical subunits, each of which contains one atom of ferric iron and a substrate binding site (Fujisawa et al., 1972a).

The enzyme has a deep red color with a broad absorption between 400 and 650 nm, which is attributable to the ferric iron bound to the enzyme (Fujisawa et al., 1972a; Fujiwara and Nozaki, 1973). Detailed kinetic analyses of the enzyme revealed that a ternary complex of oxygen, substrate, and enzyme is an obligatory intermediate of the reaction and that the degradation of this complex is the rate-limiting step of the overall reaction (Fujisawa et al., 1972b; Hori et al., 1973).

On the other hand, detailed studies on the protein structure of this class of enzymes, which are essential for the ultimate clarification of their mechanism of action, have never been done. In this paper we describe several lines of evidence to indicate that the active enzyme consists of eight identical protomers, each of which is composed of two pairs of nonidentical subunits ($\alpha_2\beta_2$).

Experimental Procedures

Biological Material. Pseudomonas aeruginosa (ATCC 23975) was grown as described previously (Fujisawa and Hayaishi, 1968). One liter of the growth medium contained: *p*-hydroxybenzoic acid, 3.0 g; NaCl, 5.0 g; $(NH_4)_2HPO_4$, 3.0 g; K_2HPO_4, 1.2 g; $FeSO_4 \cdot 7H_2O$, 0.1 g; and $MgSO_4 \cdot 7H_2O$, 0.2 g. Cells stored in a deep freezer lost little activity within 1 month.

Chemicals. Urea, purchased from Schwarz/Mann, was used without further purification in the urea treatment, and that from Wako Pure Chemical Industries, Ltd., was used after recrystallization and deionization. SP-Sephadex[1] C-25 and Sephadex G-200 were obtained from Pharmacia Fine Chemicals and Bio-Gel A-15m and A-1.5m were from Bio-Rad Laboratories. Carboxypeptidases A and B were the products of Worthington Chemicals, and carboxypeptidase Y was a kind gift from Dr. Rikimaru Hayashi of the Research Institute for Food Science, Kyoto University. Iodoacetamide, 2-mercaptoethanol, acrylamide, and sodium dodecyl sulfate were purchased from Wako Pure Chemical Industries, Ltd. Hydrazine hydrate, dimethylallylamine, phenyl isothiocyanate, and trifluoroacetic acid were obtained from Tokyo Kasei Kogyo Company, Japan. The reagents used for the automatic Edman degradation were the sequencer reagents of Wako Pure Chemical Industries, Ltd. All other chemicals were of reagent grade.

Enzyme Purification. Protocatechuate 3,4-dioxygenase was purified by the procedure of Fujisawa and Hayaishi (1968) with some modifications. All subsequent purification procedures were carried out at about 4 °C. The ammonium sulfate precipitate, obtained after streptomycin sulfate treatment, was dialyzed against 50 mM Tris-acetate buffer, pH 8.5. The turbid dialyzed enzyme preparation was passed sequentially through two columns of Bio-Gel A-15m (8 × 60 cm) and Bio-Gel A-1.5m (4 × 80 cm) in 50 mM Tris-acetate buffer, pH 8.5. The purple, clear eluate was chromatographed on a Sephadex G-200 column (7 × 60 cm) in the same buffer. During this chromatography, proteins with smaller molecular

† From the Department of Medical Chemistry, Kyoto University Faculty of Medicine, Kyoto, Japan, and the Department of Biochemistry, Osaka University School of Medicine, Osaka, Japan. *Received January 2, 1976.* This work has been supported in part by grants from the Mishima Kaiun Foundation, the Japan Foundation for Applied Enzymology, the Medical Research Foundation for Geriatric Diseases, and by a Grant-in-aid for Scientific Research from the Ministry of Education, Science and Culture, Japan.

‡ Present address: Department of Biochemistry, Hyogo College of Medicine, Nishinomiya, Hyogo, Japan.

§ Present address: Department of Biochemistry, Shiga University of Medical Science, Shiga, Japan.

[1] Abbreviations used: SP-Sephadex, sulfopropyl-Sephadex; Tris, tris(hydroxymethyl)aminomethane; Pth, phenylthiohydantoin; CPase, carboxypeptidase.

weights, including cytochromes, were separated from protocatechuate 3,4-dioxygenase which was eluted in a single peak. The fractions containing the enzyme activity were combined, 1.8 g of ammonium sulfate was added per 10 ml of the solution, and the mixture was allowed to stand for several hours. The precipitate formed was collected by centrifugation and was dissolved in about 10 ml of the buffer, followed by the addition of 1.8 g of ammonium sulfate. After repeating the ammonium sulfate precipitation procedure several times, no further precipitation occurred upon the addition of 1.8 g of ammonium sulfate per 10 ml. At this point, 2 g of ammonium sulfate per 10 ml of the solution was added with mechanical stirring. After 1 h, the precipitate was removed by centrifugation. When the supernatant solution had been allowed to stand for several days in a refrigerator, rhombic crystals appeared on the glass wall. Thus, a crystalline preparation was obtained without prior incubation with 2-mercaptoethanol, which was essential in the previous method (Fujisawa and Hayaishi, 1968). Crystalline enzyme with a specific activity of 62 units per mg of protein was obtained with a 36% yield. The crystalline preparations, thus obtained, were used throughout the present experiments unless otherwise specified.

Assay Method. The enzyme activity was routinely assayed by measuring the rate of oxygen uptake polarographically. The standard assay mixture contained, in a final volume of 2.3 ml, 150 μmol of Tris-acetate buffer, pH 7.5, 1.0 μmol of protocatechuic acid, and a suitable amount of the enzyme. One unit of enzyme activity was defined as the amount that consumed 1 μmol of the molecular oxygen, equivalent to the formation of 1 μmol of β-carboxymuconic acid, per min at 25 °C (Fujisawa and Hayaishi, 1968). The protein concentration was determined either by the absorbance at 280 and 260 nm or by dry weight as described previously (Fujisawa and Hayaishi, 1968).

Polyacrylamide Gel Electrophoresis. Disc gel electrophoresis of the native enzyme was performed according to the method of Davis (1964). A solution containing 5 mM Tris–38 mM glycine, pH 8.3, was used as the running buffer, and 7% acrylamide gel was used. Sodium dodecyl sulfate gel electrophoresis was performed according to the method of Weber and Osborn (1969). The protein samples were incubated with 10 mM sodium phosphate buffer, pH 7.0, containing 1% sodium dodecyl sulfate and 1% 2-mercaptoethanol at 37 °C for 2 h. After incubation, the sample solution was dialyzed for several hours at room temperature against 500 ml of 10 mM sodium phosphate buffer, pH 7.0, containing 0.1% sodium dodecyl sulfate and 0.1% 2-mercaptoethanol. Polyacrylamide gel containing 8 M urea was prepared by replacing water with 10 M urea in the procedure of Davis (1964). Coomassie Blue R 250 was used for staining proteins (Weber and Osborn, 1969).

Isoelectric Focusing in 6 M Urea. Isoelectric focusing of the enzyme was performed with a pH range of 3 to 10 according to the method of Vesterberg and Svensson (1966). After electrophoresis, the protein concentration and pH of each fraction eluted from the column were determined. Ultrapure urea of Schwarz/Mann was used for the electrophoresis.

Amino Acid Analysis of Proteins. The enzyme was exhaustively dialyzed against distilled water and lyophilized. The lyophilized samples were hydrolyzed with constant-boiling HCl in sealed, evacuated tubes at 110 °C for 24 and 48 h. The hydrolyzates were analyzed with an automatic amino acid analyzer, JEOL JLC-6AH. The tryptophan content was determined spectrophotometrically as described by Beavan and Holiday (1952). Cysteine was estimated separately as S-carboxymethylcysteine, after S-carboxymethylation of the protein as described below, and as cysteic acid after performic acid oxidation by the method of Moore (1963).

Preparation of S-Carboxymethylated Enzyme. Approximately 100 mg of the enzyme was dissolved in 0.2 ml of 6 M guanidine hydrochloride containing 0.25 M Tris-HCl buffer, pH 8.5, and 1 drop of 2-mercaptoethanol. The solution was flushed with nitrogen, sealed, and kept at room temperature overnight. The S-carboxymethylation was then carried out by incubation with 268 mg of iodoacetic acid for 30 min at room temperature. The reaction was terminated by the addition of 0.8 ml of 2-mercaptoethanol. The solution was diluted with 4 volumes of water and dialyzed exhaustively against water at 4 °C. The carboxymethylated enzyme was lyophilized and stored at −20 °C.

Analyses of the NH_2-Terminal Sequence. The NH_2-terminal amino acids were determined by manual Edman degradation (Edman, 1956, 1957) and by the cyanate method of Stark and Smyth (1963). Analyses of the NH_2-terminal sequence were carried out by automatic Edman degradation using a JEOL sequencer JAS-47K essentially as described in the JEOL sequencer manual. The thiazolinone derivatives of the amino acids were extracted with ethyl acetate or 1-chlorobutane. The extracted thiazolinone derivatives were converted to their Pth derivatives by the methods of Edman and Begg (1967). The Pth derivatives of Ala, Gly, Val, Pro, ΔThr, Leu, Ile, Met, and Phe were identified with a JEOL, JGC-20K gas chromatograph, equipped with a hydrogen flame ionization detector without conversion to more volatile derivatives. A column (0.2 × 200 cm) packed with Gas Chrom Q coated with 10% SE-30 was employed. The flow rate of the carrier gas was 25 ml per min and the column temperature was kept at 210 °C. Thin-layer chromatography of the Pth-amino acids was done as described by Jeppsson and Sjöquist (1967) Regeneration of the amino acids was carried out by hydrolyzing thiazolinone derivatives according to the method of Smithies et al. (1971).

Analyses of the COOH-Terminal Residue. Determination of the COOH-terminal residue was carried out either by carboxypeptidase (CPase) digestion, hydrazinolysis (Braun and Schroeder, 1967), or by the tritium labeling method.

Carboxypeptidase digestions were performed as follows: About 0.1 μmol of S-carboxymethylated protein was dissolved in sufficient 0.2 M *N*-ethylmorpholine acetate buffer, pH 8.0, containing 6 M urea, to make the final protein concentration 0.2 to 0.5%. Carboxypeptidase A or B was then added in a enzyme:substrate ratio of about 1:20 (molar basis). After various time intervals a portion of the mixture was removed and acidified to pH 2 to stop the reaction. Any resulting precipitate was removed by centrifugation and the supernatant solution was subjected to amino acid analysis. Digestion by carboxypeptidase Y was performed according to the procedure of Hayashi et al. (1973). The released amino acids were identified by an amino acid analyzer as described above.

Selective tritium labeling of the COOH-terminal amino acid was performed by the method of Matsuo et al. (1966) with some modifications. S-Carboxymethylated protein (5 mg) was dissolved in a mixture of 0.2 ml of pyridine, 50 μl or 100 μl of tritiated water (ca. 100 to 200 mCi), and 0.05 ml of acetic anhydride and incubated at 37 °C for 6 h. Since the basic subunit was sparingly soluble, 80 mg of urea was also added to the mixture. After incubation, more pyridine (0.2 ml) and acetic anhydride (0.05 ml) were added to improve the extent of tritiation. The sample containing either the whole enzyme or the acidic subunit was evaporated to dryness under reduced

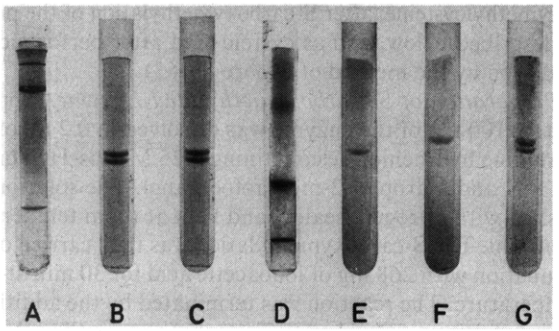

FIGURE 1: Electrophoretic pattern of protocatechuate 3,4-dioxygenase on polyacrylamide gel under various conditions. Twenty-five to fifty micrograms of proteins was applied to each gel column, and electrophoresis was performed with the positive electrode in the lower chamber. Urea treatment of the enzyme was performed as follows: the enzyme was incubated with 8 M urea in 10 mM citrate–phosphate buffer, pH 5.8, containing 0.2 M 2-mercaptoethanol, for 6 h at 37 °C. (A) The native enzyme; (B and C) sodium dodecyl sulfate gel electrophoretic patterns of the enzyme in the presence and the absence of 0.1% 2-mercaptoethanol, respectively; (D) the urea-treated enzyme; (E and F) sodium dodecyl sulfate gel electrophoretic pattern of the separated α and β subunits, respectively; (G) E + F (1:1). The bands near the bottom of the gels, A and D, are not protein bands but those of marker dye.

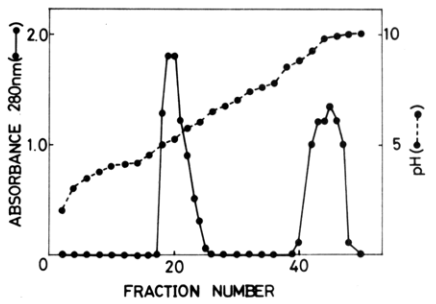

FIGURE 2: Isoelectric focusing of protocatechuate 3,4-dioxygenase in 6 M urea. A density gradient of carrier ampholite was prepared by the dense and less dense solutions; both contained 6 M urea (pH 3–10). The urea-treated enzyme (24 mg), prepared as described in Figure 1, was applied at the middle part of the column and the electrophoresis was performed at a constant voltage of 900 V for 48 h. Volume of one fraction was 1.5 ml.

pressure. The resulting residue was suspended in 0.5 ml of water and evaporated again. This washing process was repeated several times to remove exchangeable tritium. The sample containing the basic subunit was dialyzed against distilled water for 24 h and subsequently lyophilized. The dried preparations were hydrolyzed in 6 N HCl at 110 °C for 23 h. The hydrolyzates were subjected to high-voltage paper electrophoresis (2500 V, pyridine–acetic acid–H_2O (1:10:289) at pH 3.6) and to three systems of paper chromatography (Randerath, 1963) for the identification of the radioactive amino acids: I, butanol–acetic acid–H_2O (60:20:20); II, phenol–H_2O (75:25); III, phenol–ethanol–H_2O–NH_4OH (150:40:10:1). For the measurement of radioactivity, the chromatogram was cut into small pieces and counted in 10 ml of toluene scintillator (2 g of 2,5-diphenyloxazole, 0.05 g of 2,2-*p*-phenylenebis(5-phenyloxazole), and 500 ml of toluene) with a Packard Tri-Carb liquid scintillation counter, Model 3385.

Results

Disc Gel Electrophoresis. To examine the purity of the enzyme and its subunit structure, disc gel electrophoreses were performed under different conditions. The native enzyme gave a single major band and a fainter, slower moving band (Figure 1A). The faint band was not observed when the sample was incubated with 10 mM each of iodoacetamide and 2-mercaptoethanol for a few days, or when electrophoresis was carried out in the presence of sodium dodecyl sulfate, indicating that the faint band was a polymerized form of the enzyme.

When the native enzyme was treated with sodium dodecyl sulfate and 2-mercaptoethanol as described under Experimental Procedures, two distinct bands, which stained equally, were observed on sodium dodecyl sulfate gel electrophoresis (Figure 1B), suggesting that the enzyme consists of nonidentical subunits. The molecular weights of these subunits were estimated to be 22.5×10^3 and 25×10^3, respectively, using cytochrome *c* (11 700), trypsin (23 300), lactic dehydrogenase (36 000), aldolase (40 000), ovalbumin (43 000), catalase (60 000), and serum albumin (68 000) as protein standards.

When the incubation and electrophoresis were carried out in the absence of mercaptoethanol, an essentially identical result was obtained (Figure 1C), indicating that disulfide linkages are not involved in the interaction of these subunits. When the enzyme treated with 8 M urea was subjected to disc gel electrophoresis in the absence of sodium dodecyl sulfate, it also gave two clearly separated bands (Figure 1D).

Isoelectric Focusing in 6 M Urea. On isoelectric focusing in the presence of 6 M urea, the enzyme was separated into two major fractions with isoelectric points of approximately 5.2 and 9.5, respectively (Figure 2). Both the acidic and basic fractions, hereafter referred to as α and β subunits, respectively, gave a single band on sodium dodecyl sulfate gel electrophoresis (Figure 1E,F). The α subunit corresponded to the 22 500-dalton band, and the β subunit, to the 25 000-dalton band. Sodium dodecyl sulfate gel electrophoresis of a mixture of equal amounts of the two fractions gave two distinct bands which were stained equally (Figure 1G). The absorbances at 280 nm of 1% solutions of the α and β subunits were 1.062 and 0.971, respectively, which were experimentally obtained by measurements of absorbance and dry weight. Using these values, yields of the α and β subunits were calculated to be 10.7 mg (0.476 μmol) and 11.2 mg (0.448 μmol), respectively. Total recovery of the protein was approximately 91% and the molar ratio of the α and β subunits obtained was roughly 1:1.

Separation of Subunits by SP-Sephadex Chromatography. The α and β subunits were separated by SP-Sephadex C-25 chromatography in the presence of 6 M urea at pH 5.8. One fraction was not absorbed on the column and eluted with the equilibrating buffer, while the second fraction was eluted with 0.5 M NaCl (Figure 3). On the first chromatography (Figure 3A), the first fraction showed a shoulder. Examination by sodium dodecyl sulfate gel electrophoresis revealed that the main peak of the first fraction contained mainly the α subunit and the shoulder contained a mixture of the α and β subunits, while the second fraction contained mostly the β subunit. The small absorbing peaks in Figure 3A which appeared just prior to the introduction of the 0.5 M NaCl were not due to protein because these peaks appeared even during chromatography of a non-protein-containing control and the fractions showed no Coomassie blue staining band on sodium dodecyl sulfate gel electrophoresis. When the first fraction was dialyzed against 6 M urea in 10 mM citrate–phosphate buffer, pH 5.8, containing 10 mM 2-mercaptoethanol, and rechromatographed on the SP-Sephadex C-25 column, the β subunit was completely separated from the α subunit (Figure 3B).

The yield of the α subunit which was calculated from the elution pattern of the second chromatography was 21.6 mg (0.96 μmol) and that of the β subunit was 22.4 mg (0.90 μmol),

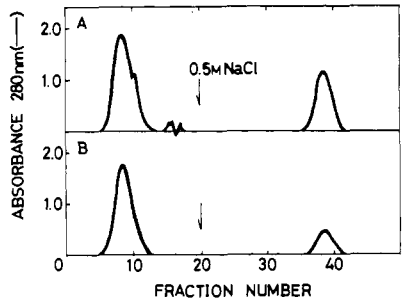

FIGURE 3: Separation of protocatechuate 3,4-dioxygenase subunits by SP-Sephadex chromatography. The urea-treated enzyme (50 mg), prepared as described in Figure 1, was applied to a SP-Sephadex C-25 column (2 × 30 cm), equilibrated with 6 M urea in 10 mM citrate-phosphate buffer, pH 5.8, containing 10 mM 2-mercaptoethanol. After the first protein fraction was eluted, the column was eluted with the same buffer containing 0.5 M NaCl. (A) the first chromatographic pattern; (B) the first peak from Figure 3A was dialyzed overnight against 6 M urea in 10 mM citrate-phosphate buffer, pH 5.8, containing 10 mM 2-mercaptoethanol at room temperature and then rechromatographed under the same conditions. Volume of one fraction was 12 ml.

which was the sum of the second peaks of the first and second chromatographies. Total recovery of the protein was 88% and the molar ratio of the α and β subunits obtained was approximately 1:1.

Each fraction, which gave a single band on sodium dodecyl sulfate gel electrophoresis, was exhaustively dialyzed against distilled water at 4 °C and lyophilized. The lyophilized powder was used for the determination of the amino acid composition.

Amino Acid Composition. The results of amino acid analyses of the α and β subunits showed a distinct difference between the two subunits (Table I). After separation by SP-Sephadex chromatography, each isolation protein was dialyzed against distilled water for a few days and lyophilized. Each protein was dissolved in constant-boiling HCl and hydrolyzed as described under Experimental Procedures. The results are expressed as number of residues on the basis of the assumption that the native enzyme has a molecular weight of 700 000, the α subunit, 22 500, and the β subunit, 25 000. Values are the averages of several determinations, including those of the S-carboxymethylated samples. The amino acid composition of the native enzyme obtained by direct analyses agreed with that calculated from analyses of the isolated α and β subunits, based on the assumed subunit structure of $(\alpha_2\beta_2)_8$.

NH_2-Terminal Residues. The NH_2-terminal residues of the native enzyme and the α and β subunits were all found to be proline, with recoveries of 68, 61, and 60%, based on molecular weights of 700 000, 22 500, and 25 000, respectively. Essentially the same results were obtained with the cyanate method. These results were also confirmed by the NH_2-terminal sequence analyses.

NH_2-Terminal Sequences. Eight cycles of automatic Edman degradation on the native enzyme were performed (Table II). In each of the degradation steps, except for the first and third steps, a pair of amino acids was obtained, suggesting that the enzyme consists of two nonidentical polypeptide chains. NH_2-terminal sequence analyses were also performed on the separated α and β subunits for 14 steps, and the yields of amino acid released are included in Table II. Amino acids released from the α and β subunits at each step coincided with those released from the native enzyme.

COOH-Terminal Residues. Determination of the COOH-terminal residue using three different methods, digestion by carboxypeptidases, hydrazinolysis, and tritium labeling, resulted in the same answer (Table III).

In both the native enzyme and the α subunit, phenylalanine was the only amino acid released from the protein in a significant amount by either carboxypeptidases A, B, or Y. Likewise, only phenylalanine was found by hydrazinolysis for both the native enzyme and the α subunit. Selective tritium labeling of the COOH-terminal amino acid was performed as described under Experimental Procedure. Upon high-voltage paper electrophoresis of the acid hydrolyzates (22 500 cpm) of the native enzyme, about 60% of the total radioactivity was re-

TABLE I: Amino Acid Compositions of Native Protocatechuate 3,4-Dioxygenase and Its Subunits.[a]

	α	β	$(\alpha_2 + \beta_2) \times 8$	Native[b]
Lys	5	8	208	182
His	5	7	192	194
Arg	11	14	400	351
Asp	24	23	752	718
Thr[c]	10	9	304	286
Ser[c]	5	9	224	205
Glu	24	16	640	513
Pro	13	21	544	477
Gly	14	18	512	460
Ala	19	15	544	477
Val	11	9	320	265
Met	1	2	48	46
Ile	12	15	432	364
Leu	19	17	576	459
Tyr	7	8	240	188
Phe	9	9	288	247
½-Cys[d]	2	4	96	95
Trp	6	4	160	160

[a] Unless otherwise indicated, the values for the amino acids represented an average of at least four sets of analyses. [b] Data were taken from Fujisawa and Hayaishi (1968). [c] These values were extrapolated to zero-time hydrolysis. [d] Also determined as cysteic acid.

TABLE II: The NH_2-Terminal Sequences and Yields of the Native Enzyme and Its Subunits.[a]

Step	Native Enzyme	α Subunit	β Subunit
1	Pro (22.2)	Pro (0.61)	Pro (0.59)
2	Ile (9.0), Ala (15.0)	Ile (0.66)	Ala (0.63)
3	Glx (24.4)	Glu (0.94)	Gln (0.73)
4	Leu (7.3), Asx (12.4)	Leu (0.66)	Asp (0.72)
5	Leu (7.3), Asx (11.1)	Leu (0.66)	Asn (0.66)
6	Pro (7.9), Ala (5.7)	Pro (0.41)	Ala (0.37)
7	Glx (3.8), Arg (1.7)	Glu (0.38)	Arg (0.24)
8	Thr (3.9), Phe (1.0)	Thr (0.35)	Phe (0.20)
9		Pro (0.39)	Val (0.30)
10		Ser (0.31)	Ile (0.29)
11		Glx (0.44)	Arg (0.18)
12		Thr (0.25)	Asx (0.32)
13		Ala (0.45)	Arg (0.13)
14		Gly (0.4)	Asx (0.21)

[a] The carboxymethylated native enzyme (0.0082 μmol), α subunit (0.22 μmol), and β subunit (0.2 μmol) were used. The sequential determination was performed by a sequencer as described under Experimental Procedures. Yields were calculated from the results of amino acid analyses after HI hydrolysis and are given in parentheses as residues per mole of protein.

TABLE III: Determination of the COOH-Terminal Residue by Various Methods.[a]

Methods		Native	α Subunit	β Subunit
Carboxypeptidase A	1 h	Phe (10.1) Glu (tr) Asp (tr) Ala (tr)	Phe (0.425) Glu (tr) Asp (tr)	Glu (tr) Phe (tr) Ala (tr)
	5 h	Phe (11.5) Glu (1.5) Asp (0.7) Ala (tr)	Phe (0.53) Glu (0.18) Asp (tr) Ala (tr)	Glu (tr) Phe (tr) Ala (tr)
	24 h	Phe (13.4) Glu (2.5) Asp (1.3) Ala (1.1)	Phe (0.72) Glu (0.3) Asp (0.18) Ala (0.17)	Glu (tr) Phe (tr) Ala (tr)
Carboxypeptidase Y	0.5 h	Phe (15.0)	Phe (0.43)	Phe (tr)
Hydrazinolysis		Phe (12.4)	Phe (0.6)	ND
Tritium labeling[b]		Phe	Phe	ND

[a] Yields of the amino acid are given in parentheses as residues per mole of protein. [b] Labeled amino acids were identified qualitatively by paper chromatography.

covered in the neutral amino acid fraction, with about 21 and 10% in the acidic and basic amino acid fractions, respectively. When an aliquot (7650 cpm) of the neutral amino acid fraction was subjected to paper chromatography with the solvent system I, radioactivity was found only in phenylalanine with a recovery of 62%. Likewise, with the α subunit, only phenylalanine was found to be labeled significantly with a recovery of radioactivity of about 62% in the neutral amino acid fraction and with about 52% of this in phenylalanine. On the other hand, the COOH-terminal residue of β subunit was not detected by any of these methods.

Discussion

Protocatechuate 3,4-dioxygenase has been reported to consist of eight identical subunits (Fujisawa et al., 1972a,b). However, data presented in this paper clearly demonstrated that the subunit was further dissociated into two nonidentical, smaller subunits. Treatment of the enzyme with 0.05 N NaOH–0.2 M NaCl brought about the dissociation of the enzyme into homogeneous subunits with a molecular weight in the range between 90 000 and 120 000 (Fujisawa et al., 1972a), whereas the treatment with sodium dodecyl sulfate or urea gave two nonidentical subunits, α and β, with molecular weights of approximately 22 500 and 25 000, respectively. Since the presence of 2-mercaptoethanol did not affect the dissociation with sodium dodecyl sulfate, disulfide linkages seemed not to be involved in the interaction of the subunits. The crystalline preparation of the enzyme gave a single band on polacrylamide gel electrophoresis, and the treatment of the enzyme by sodium dodecyl sulfate brought about its total conversion to the small subunits as shown in Figure 1. From these results, the possibility that the dissociation is due to a nonspecific proteolytic digestion during the purification seems highly unlikely.

It is of interest that the NH_2-terminal residues of the two nonidentical subunits were both determined to be proline, which occurs only rarely as the NH_2-terminal residue, especially in bacterial enzymes (Waller, 1963; Capecchi, 1968). Recovery of the NH_2-terminal proline from the native enzyme was 22.2 residues per mole of enzyme. This provides additional evidence that the enzyme consists of more than eight polypeptide chains.

The enzyme contains eight atoms of ferric iron and eight substrate binding sites per mole of enzyme, based on the molecular weight of 700 000 and consists of eight identical protomers (Fujisawa et al., 1972a). The molar ratio of the separated α and β subunits obtained from the urea-treated enzyme by means of either isoelectric focusing or SP-Sephadex chromatography was approximately 1:1. These results together with the fact that the calculated amino acid composition, based on the assumed subunits structure of $(\alpha_2\beta_2)_8$, agreed quite well with that obtained by direct analyses of the native enzyme confirm the assumption that the native enzyme consists of eight protomers, each of which is composed of a pair of two nonidentical subunits ($\alpha_2\beta_2$). Although we have not succeeded in obtaining active protomers with a molecular weight of 90 000, the protomer appears to contain one atom of iron and to form an active site of the enzyme. Clarification of the structure of the active site requires further investigation. Although the COOH-terminal residue of the α subunit was found to be phenylalanine by three different methods, that of the β subunit was not detected by any of these methods. The reason for this is now under investigation. The facts that the α and β subunits contained one and two methionine residues, respectively, and that no methionine was released by Edman degradation up to 14 steps may facilitate the determination of the total amino acid sequence of these subunits. Work in this direction is now in progress in this laboratory.

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Lysine-Sensitive Aspartokinase of *Escherichia coli* K12. Synergy and Autosynergy in an Allosteric V System†

Jean-Pierre Mazat* and Jean-Claude Patte‡

ABSTRACT: The interactions of the lysine-sensitive aspartokinase of *E. coli* K12 with lysine and leucine, as evidenced in the inhibition and binding curves, are well explained by the equations of an allosteric V model. Mathematical treatments of such a model lead to new linearized plots. These representations are applied to our experimental results and allow the direct determination of some parameters of the model (equilibrium constant L' and leucine dissociation constants). The other parameters are obtained by an optimization method. The theoretical curves drawn according to this model account well for the synergistic inhibition between lysine and leucine and for the role of the two nonequivalent lysine binding sites ("autosynergy").

The lysine-sensitive aspartokinase of *Escherichia coli* K12 (aspartokinase III)[1] is one of the three enzymes catalyzing the phosphorylation of the β-carboxyl of aspartate in this bacteria; L-lysine is the feedback inhibitor of this activity. Lysine inhibition is cooperative (Stadtman et al., 1961; Patte et al., 1967).

It has been shown that the protein is a dimer of mol wt 105 000 composed of two identical subunits (Richaud et al., 1973). There are two nonequivalent binding sites for lysine per subunit, i.e., four lysine sites/enzyme molecule (Richaud et al., 1974).[2] The binding of lysine is cooperative (Richaud et al., 1974).

Nonspecific amino acids such as leucine, isoleucine, etc., also substantially inhibit the enzymatic activity; a synergistic inhibition is observed between lysine and these amino acids (Patte et al. 1965). The leucine binding site(s) appears different from the two lysine sites (Richaud et al., 1974) (as their number is not experimentally measurable, the simplest assumption, that there exists only one leucine binding site per subunit, i.e., two leucine sites/enzyme molecule, will be used in the following).

Monod et al. (1965) have proposed aspartokinase III as an allosteric V system for the following reasons: no cooperative effect of substrates is observed, and lysine and leucine are strictly noncompetitive inhibitors toward aspartate (Stadtman et al., 1961; Truffa-Bachi et al., 1966; Wampler and Westhead, 1968).

This hypothesis was further strengthened by recent results of our laboratory: the sigmoidicity of the lysine binding curve disappears when binding is performed in the presence of leucine, a synergistic inhibitor (Richaud et al., 1974). This could be explained by a shift of an allosteric equilibrium (Monod et al., 1965). Moreover, physical techniques suggest the existence of different forms for the protein, depending on the presence of the inhibitory ligands (Richaud et al., 1974).

In this paper we will show that an allosteric V model explains quantitatively both kinetic and binding results obtained with the inhibitory ligands, and particularly the synergistic inhibition. The parameters of the model (dissociation constants for the inhibitors and equilibrium constants) will be estimated.

Materials and Methods

Strains Used and Enzyme Purification. Aspartokinase III was purified from *E. coli* K12 strain D06 according to the previously published procedure (Lafuma et al., 1970; Richaud et al., 1973). For kinetic measurements, the partially pure fraction, obtained after the DEAE-Sephadex step, was used (sp act. 5550 nmol min^{-1} mg^{-1}).

† From the Laboratoire de Biochimie B.B.C., Université de Bordeaux II, 33405 Talence, France (J.-P.M.) and Institut de Microbiologie, Université de Paris-Sud, 91405 Orsay, France (J.-C. P.). *Received February 3, 1976.* Financial support came from the Centre National de la Recherche Scientifique, the Delegation Générale à la Recherche Scientifique et Technique and the Fondation pour la Recherche Médicale Française. This work is part of the Thesis to be submitted by J. P. Mazat, in partial fulfillment of the requirements for the Doctorat-ès-Sciences. A preliminary report of this work has been given at the 9th FEBS Meeting (Budapest, 1974).

[1] Abbreviations used are: AK III or aspartokinase III, L-lysine-sensitive aspartokinase of *E. coli* K12 (EC 2.7.2.4); DEAE, diethylaminoethyl.

[2] Recently Funkhouser et al. (1974) have obtained, in the case of aspartokinase III of *E. coli* B, one binding site only for lysine per enzyme molecule; however, it is not known whether the subunits of this enzyme are identical. Also, saturation of binding is not reached in their experiments.