Biochimica et Biophysica Acta, 527 (1978) 171—181

BBA 68594

STUDIES ON THE REACTION INTERMEDIATE OF PROTOCATECHUATE 3,4-DIOXYGENASE

FORMATION OF ENZYME-PRODUCT COMPLEX

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(Received April 13th, 1977)

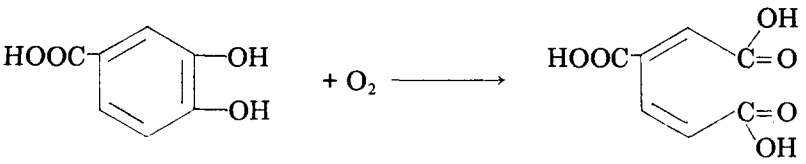
Summary

The nature of the oxygenated intermediate observed (Fujisawa, H., Hiromi, K., Uyeda, M., Okuno, S., Nozaki, M. and Hayaishi, O. (1972) J. Biol. Chem. 247, 4422—4428) during the reaction of protocatechuate 3,4-dioxygenase (protocatechuate:oxygen 3,4-oxidoreductase (decyclizing), EC 1.13.11.3) was investigated. 3,4-Dihydroxyphenylpropionic acid and 3,4-dihydroxyphenylacetic acid were used as substrates of the enzyme to slow down the rate of the reaction. The enzyme reactions were performed under conditions where the concentration of the organic substrate was lower than those of the enzyme and oxygen in the reaction mixture. The reactions were stopped before completion by the addition of hydrochloric acid or guanidine hydrochloride and then the organic compounds were extracted from the reaction mixture to be analyzed. The qualitative analyses by thin-layer chromatography revealed that there was no species other than the organic substrate and the enzymatic reaction end-product during reaction. The quantitative spectrophotometric analyses revealed that the organic substrate which had participated in the formation of the oxygenated intermediate existed as a species indistinguishable from the reaction end-product, indicating that the oxygenated intermediate was not a simple complex of oxygen, substrate and the enzyme, i.e., a ternary complex, but a species rather close to a binary complex of product and the enzyme.

Introduction

Protocatechuate 3,4-dioxygenase (protocatechuate:oxygen 3,4-oxidoreductase (decyclizing), EC 1.13.11.3) catalyzes the cleavage of the benzene ring of protocatechuic acid with the incorporation of two atoms of molecular oxygen, resulting in the formation of β-carboxymuconic acid [1]. It is a red, non-

heme iron protein with a



broad optical absorption between 400 and 650 nm [2]. It has been shown to have a molecular weight of 700 000 and to be composed of eight identical subunits, each of which contains one atom of iron and one substrate binding site [3]. The trivalent iron in the enzyme participates in the reaction as an essential cofactor [3—5].

Involvement of a ternary complex of oxygen, organic substrate and enzyme has been proposed in some dioxygenase-catalyzed reactions [6—10]. Kinetic and spectral studies with protocatechuate 3,4-dioxygenase revealed that the enzyme first combines with the organic substrate to form a binary complex and then reacts with oxygen to form a ternary complex which decomposes to an oxygenated end-product and the enzyme [11—13]. The nature of the ternary complex, i.e., the oxygenated intermediate, however, remains to be analyzed.

In order to study the reaction mechanism of the enzyme, the chemical structure of the organic substrate in the oxygenated intermediate was examined. Since the velocity of the reaction of the enzyme with protocatechuic acid was too fast to be analyzed, substrate analogs such as 3,4-dihydroxyphenylpropionic acid and 3,4-dihydroxyphenylacetic acid were used to slow down the rate of the reaction.

Materials and Methods

Chemicals. 3,4-Dihydroxyphenylpropionic acid was obtained from K & K Laboratories. 3,4-Dihydroxyphenylacetic acid was a product of Sigma. Precoated silica gel glass plates 60 F_{254} were obtained from E. Merck.

Biological materials. Crystalline preparation of protocatechuate 3,4-dioxygenase was prepared from extracts of *p*-hydroxybenzoate-induced cells of *Pseudomonas aeruginosa* (ATCC 23975) as described previously [2,3].

Determination of enzyme concentration. The protein concentration was determined by measuring the absorbance at 280 nm, taking 13.2 as the absorbance of a 1% solution at pH 8.5 [2]. The molar concentration of protocatechuate 3,4-dioxygenase was expressed as the concentration of active sites of the enzyme, based on the results that the enzyme had a molecular weight of 700 000 and was composed of eight identical subunits, each containing one substrate binding site [3].

Measurement of spectral change and oxygen consumption. Absorption spectra were recorded with a Union spectrophotometer SM 401, Union Giken Co. (Hirakata, Japan) or a Shimadzu recording spectrophotometer UV-200, Shimadzu Co. (Kyoto, Japan). The Union spectrophotometer SM 401 was equipped with a small oxygen electrode for simultaneous measurements of spectral change and oxygen consumption.

Results and Discussion

Time course of amounts of oxygenated intermediate during enzyme reaction with 3,4-dihydroxyphenylpropionic acid

The formation and decomposition of the oxygenated intermediate with 3,4-dihydroxyphenylpropionic acid were followed at 433 nm, the isosbestic point of the enzyme and enzyme-substrate complex [11]. To convert most substrate added to the reaction mixture to the intermediate, an excess amount of the enzyme was added. The reaction was carried out at 5°C to slow down the reaction rate. Fig. 1 represents the rate of oxygen consumption and the formation and decomposition of the oxygenated intermediate. They were recorded simultaneously with a spectrophotometer equipped with an oxygen electrode. A rapid decrease in the absorbance at 433 nm was observed in the early stage of the reaction followed by a gradual restoration to the original level. The rate of the oxygen consumption was compatible with the rapid decrease in the absorbance at 433 nm, supporting our previous observation that this spectral species was the oxygenated intermediate [11]. At 80 s after the start of the reaction, i.e., at the time of maximum formation of the intermediate, the amount (0.172 mM) of the oxygen consumed in the reaction corresponded to 86% of the organic substrate added to the reaction mixture, indicating that 86% of the substrate was accumulated as the oxygenated intermediate at 80 s.

While the above experiment was performed at a fixed concentration of the organic substrate, similar experiments were carried out under different initial concentrations of 3,4-dihydroxyphenylpropionic acid in the presence of an excess amount of oxygen. The maximum changes in the absorbance at 433 nm

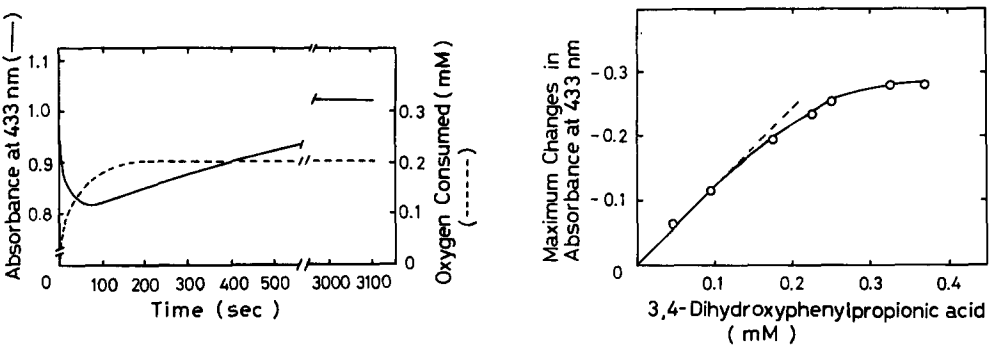


Fig. 1. Time-course for the rates of oxygen consumption and absorption at 433 nm during enzyme reaction with 3,4-dihydroxyphenylpropionic acid as a substrate. Concentrations of protocatechuate 3,4-dioxygenase, 3,4-dihydroxyphenylpropionic acid, oxygen and Tris-HCl buffer, pH 8.5, were 0.312 mM, 0.200 mM, 0.38 mM and 50 mM, respectively, in a final volume of 3.0 ml. The reaction was started by the addition of 3,4-dihydroxyphenylpropionic acid at 5°C. Oxygen consumption (- - - - -) and absorption at 433 nm (———) were recorded simultaneously.

Fig. 2. Effects of concentrations of 3,4-dihydroxyphenylpropionic acid as a substrate on maximum change in absorbance at 433 nm. Concentrations of protocatechuate 3,4-dioxygenase, oxygen and Tris-HCl buffer, pH 8.5, were 0.270 mM, 0.38 mM and 50 mM, respectively. Experiments were carried out as described in the legend for Fig. 1, with different concentrations of 3,4-dihydroxyphenylpropionic acid. The maximum changes in absorbance were plotted against the concentrations of 3,4-dihydroxyphenylpropionic acid.

at different concentrations of the organic substrate were measured. When these values were plotted against the concentrations of the organic substrate, a linear relationship was obtained under the condition that the concentration of the organic substrate was lower than those of the enzyme and oxygen in the reaction mixture as shown in Fig. 2. The molar absorptivity of the decrease in optical absorption at 433 nm due to the formation of the oxygenated intermediate was calculated to be approx. 1200, in good agreement with the value, 1130, calculated from our previous finding that a conversion of 0.387% solution of the enzyme to the oxygenated intermediate caused a decrease of 0.050 absorbance at 433 nm [11]. The concentration of the oxygenated intermediate accumulated at 80 s in the experiment shown in Fig. 1 was estimated to be approx. 0.167 mM from the molar absorption difference between the enzyme and the oxygenated intermediate. This value was in good agreement with the amount of oxygen consumed. When the enzyme protein was precipitated by the addition of either ammonium sulfate (70% saturation) or acetone (90%) at 80 s in separate experiments under the same conditions, a significant amount of compounds including the end-product was not detected spectrophotometrically in the supernatant solution. These results indicated that almost all consumed oxygen participated in the formation of the intermediate and there was no liberation of the oxygenated end-product from the oxygenated intermediate at 80 s in the experiment shown in Fig. 1.

Absorption spectra of 3,4-dihydroxyphenylpropionic acid and its oxygenated end-product

The ultraviolet absorption spectrum of 3,4-dihydroxyphenylpropionic acid had a maximum at 280 nm, as shown in Fig. 3A. Oxidation of 3,4-dihydroxyphenylpropionic acid with the ring opening between the adjacent hydroxyl groups, analogous to the known chemical [14] and enzymatic [15,16] oxidation of catechol to *cis*, *cis*-muconic acid, should yield β-carboxyethylmuconic

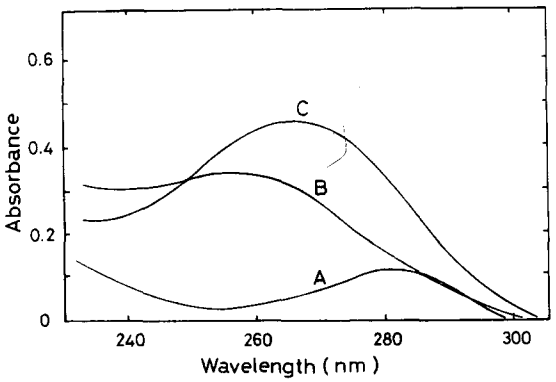


Fig. 3. Absorption spectra of the reaction product with 3,4-dihydroxyphenylpropionic acid as a substrate. Concentrations of protocatechuate 3,4-dioxygenase, 3,4-dihydroxyphenylpropionic acid, and Tris-HCl buffer, pH 7.5, were 1.20 μM, 39.8 μM and 50 mM, respectively, in a final volume of 3.0 ml. The reaction was started by the addition of the enzyme at room temperature. After incubating for 30 min, the reaction mixture was acidified to pH 2 with 2 M HCl. Curve A, before addition of the enzyme; curve B, before acidification; curve C, after acidification. The spectra were corrected for the dilution of the sample.

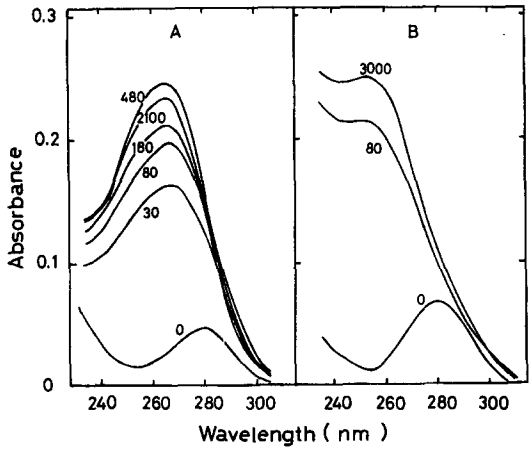


Fig. 4. Spectral changes of reaction products during the reaction with 3,4-dihydroxyphenylpropionic acid as a substrate. Concentrations of protocatechuate 3,4-dioxygenase, 3,4-dihydroxyphenylpropionic acid, oxygen and Tris-HCl buffer, pH 8.5, were 0.312 mM, 0.200 mM, 0.38 mM and 50 mM, respectively, in a final volume of 0.5 ml. The reaction was initiated by the addition of the organic substrate at 5°C and stopped by two different methods described below. Numerals in the figure represent the incubation time in seconds. A, enzyme reaction was stopped by acidification to pH 2 with 0.1 M HCl. The reaction products were extracted with 4 ml of ethyl acetate and evaporated to dryness at 0°C. The dried residue was taken up in 0.5 ml of ethyl acetate and then 0.2 ml of the resulting sample was subjected to thin-layer chromatography developed in benzene/methanol/acetic acid (8 : 4 : 1, v/v). The remaining sample was evaporated again and dissolved in 2.5 ml of water for measurement of the absorption spectrum. B, enzyme reaction was stopped by the addition of 2.5 ml of 7 M guanidine-HCl solution, pH 7. The enzyme protein was removed by ultrafiltration with a Diaflo membrane (PM 10, Amicon Co.) and the absorption spectrum of the filtrate was recorded. Blank solutions for A and B were obtained by the similar experiments except for the omission of the organic substrate in the incubation mixture.

acid of *cis-cis* configuration. The absorption spectrum of the enzymatic reaction end-product of 3,4-dihydroxyphenylpropionic acid, presumably β-carboxy-*cis*, *cis*-muconic acid, had a maximum at 256 nm as shown in Fig. 3B. When the reaction product was acidified to pH 2 with 2 M HCl, its spectrum was altered and reached a stable value within several minutes. The resulting new spectrum had a maximum at 265 nm as shown in Fig. 3C. The shift of the absorption maximum from 256 to 265 nm after acidification was very similar to that observed in a *cis* → *trans* isomerization of β-carboxymuconic acid, the reaction end-product with protocatechuic acid as a substrate [16].

Qualitative analyses of reaction products

In order to characterize the nature of the substrate in the transient ternary complex, the compounds extracted from the complex during the reaction were analyzed spectrophotometrically (Fig. 4) and chromatographically. Since the experimental conditions for the reaction employed in these experiments were similar to those described in Fig. 1, most of the substrate added to the reaction mixture should participate in the formation of the intermediate at 80 s after the start of the reaction. The fact that the absorption spectra of the compounds extracted from the intermediate at 80 s were similar to those of the reaction end-product extracted from the reaction mixture after completion of the reaction, as shown in Fig. 4, suggests that the substrate incorporated into

the oxygenated intermediate had already been converted to a species indistinguishable from the reaction end-product. The absorption spectrum of the reaction end-product obtained after acidification at 2100 s had a maximum at 265 nm as shown in Fig. 4A and was very similar to the spectrum shown in Fig. 3C.

R_F values after thin-layer chromatography 3,4-dihydroxyphenylpropionic acid and β-carboxyethylmuconic acid were determined to be 0.82 and 0.53, respectively. At 80 s, when most of the substrate added to the reaction mixture participated in the formation of the oxygenated intermediate and no end-product was liberated as described previously, the major component extracted from the reaction mixture was identified as the end-product. No species other than the substrate and the end-product were detected during reaction. Thin-layer chromatography developed in *n*-butanol/acetic acid/water (10 : 2 : 1, v/v) gave essentially the same result.

Similar spectral changes were observed in the experiment stopped by the addition of trichloroacetic acid (final concentration, 4%) in place of HCl. The spectra of the reaction products obtained after the treatment with 6 M guanidine hydrochloride under neutral conditions at 3000 s had a maximum at 256 nm (Fig. 4B) and it was very similar to the spectrum shown in Fig. 3B.

Quantitative analyses of reaction products

Since spectrophotometric analysis (Fig. 4) and thin-layer chromatography revealed that there was no species other than the substrate and the end-product during reaction, the amounts of the substrate and the end-product during the reaction were calculated from the absorption spectra shown in Fig. 4. When the reaction was stopped by the addition of guanidine hydrochloride under neutral conditions, the spectrum of the enzymatic end-product, β-carboxyethyl-*cis*, *cis*-muconic acid, had a broad peak at 256 nm with a molar extinction coefficient of 9000. The end product had an absorption at 280 nm with a molar extinction coefficient of 3700. The similarly treated substrate had an absorption at 256 nm with a molar extinction coefficient of 430 and an absorption peak at 280 nm with a molar extinction coefficient of 2400. The amounts of each in mixture during reaction were calculated from absorbance values at 256 and 280 nm of each curve shown in Fig. 4B (Table I, Expt. 2). Acidification to pH 2 caused a rapid *cis* → *trans* isomerization of the end-product as discussed before and the spectrum of the end product extracted from such acidified reaction mixture had a maximum at 265 nm with a molar extinction coefficient of 12 500. The similarly treated substrate had an absorption at this wavelength with a molar extinction coefficient of 1130, and an absorption peak at 280 nm with a molar extinction coefficient of 2640. The *trans* isomer of the end-product had an absorption at 280 nm with a molar extinction coefficient of 9000. The amounts of each in mixtures during reaction were calculated from absorbance values at 265 and 280 nm of each curve shown in Fig. 4A (Table I, Expt. 1). As shown in Table I, at early stages of the reaction, i.e., at 30 or 80 s after the start of the reaction, the rate of the formation of the end-product was very close to those of both the formation of the oxygenated intermediate and oxygen consumption. Since there was no release of the end-product from the enzyme at this stage as described before, both the end-product accumulated and the oxygen consumed during the reaction accounted for the amount of the

TABLE I

AMOUNTS OF THE END-PRODUCT DURING REACTION CALCULATED FROM THE ABSORPTION SPECTRA SHOWN IN FIG. 4

The amounts of the substrate and the end-product in reaction mixtures were calculated from the absorbance values of the spectra shown in Fig. 4, based on each extinction coefficient, as described in the text.

Reagent used to stop reaction	Reaction time (s)	Substrate calculated (nmol)	End-product calculated (nmol)	Sum * (nmol)	End-product in sum (%)	Oxygenated ** intermediate (%)	O_2 *** consumed (%)
Expt. 1: HCl	0	48	0	48	0	0	0
	30	13	31	44	70	69	63
	80	5	40	45	89	83	86
	180	1	46	47	98	71	100
	480	1	51	52	99	48	100
	2100	0	48	48	100	0	100
Expt. 2: Guanidine-HCl	0	82	0	82	0	0	0
	80	10	66	76	87	83	86
	3000	0	82	82	100	0	100

* The overall yields were more than 70%, calculated from the amount of the substrate added to the reaction mixture.

** Expressed as percentage of the amount of the substrate added to the reaction mixture. The amount of the oxygenated intermediate was calculated from the decrement in absorbance at 433 nm shown in Fig. 1, taking the value of 1200 of an extinction coefficient.

*** Estimated from the results shown in Fig. 1.

oxygenated intermediate. At subsequent stages of the reaction, i.e., at 180 or 480 s after the start of the reaction, some of the oxygenated intermediate had yielded the free end-product and the rest remained unchanged. After completion of the reaction, i.e., at 2100 or 3000 s, all of the oxygenated intermediate had been converted to the free end-product and the enzyme. These quantitative analyses provided further evidence to support the contention that the organic substrate which had participated in the formation of the oxygenated intermediate existed as a species indistinguishable from the reaction end-product.

Reaction of protocatechuate 3,4-dioxygenase with 3,4-dihydroxyphenylacetic acid

The nature of the transient oxygenated intermediate of protocatechuate 3,4-dioxygenase with 3,4-dihydroxyphenylacetic acid as a substrate was also studied. In Fig. 5 is shown the time-course for the formation and decomposition of the oxygenated intermediate with 3,4-dihydroxyphenylacetic acid followed at 450 nm, the isosbestic point of the enzyme and enzyme-substrate complex [11]. When the maximum changes in absorbance at 450 nm were plotted against the concentrations of the organic substrate, a linear relationship was obtained under the condition that the concentration of the organic substrate was lower than those of the enzyme and oxygen in the reaction mixture as shown in Fig. 6. The molar absorptivity of the decrease in absorbance at 450 nm was calculated to be approx. 1000, in good agreement with the value of 1150 calculated from our previous finding that a conversion of 0.387% solution of the enzyme to the oxygenated intermediate caused a decrease of 0.051 absorbance units at 450 nm [11]. The concentration of the oxygenated intermediate accumulated at 20 s after the start of the reaction in the experiment

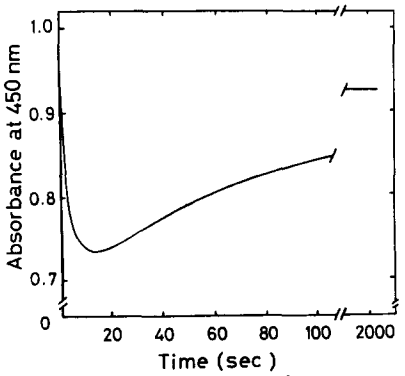


Fig. 5. Time-course of absorption at 450 nm during reaction with 3,4-dihydroxyphenylacetic acid as a substrate. Concentrations of protocatechuate 3,4-dioxygenase, 3,4-dihydroxyphenylacetic acid, oxygen and Tris-HCl buffer, pH 8.5, were 0.270 mM, 0.195 mM, 0.38 mM and 50 mM, respectively, in a final volume of 3.0 ml. The reaction was started by the addition of 3,4-dihydroxyphenylacetic acid at 5° C.

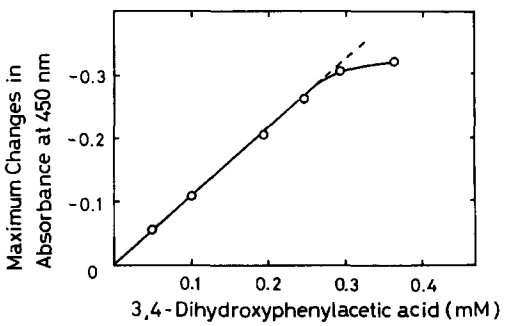


Fig. 6. Effects of concentrations of 3,4-dihydroxyphenylacetic acid as a substrate on maximum change in absorbance at 450 nm. Concentrations of protocatechuate 3,4-dioxygenase, oxygen, and Tris-HCl buffer, pH 8.5, were 0.270 mM, 0.38 mM and 50 mM, respectively. Experiments were carried out as described in the legend for Fig. 5, with different concentrations of 3,4-dihydroxyphenylacetic acid. The maximum changes in absorbance were plotted against the concentrations of 3,4-dihydroxyphenylacetic acid.

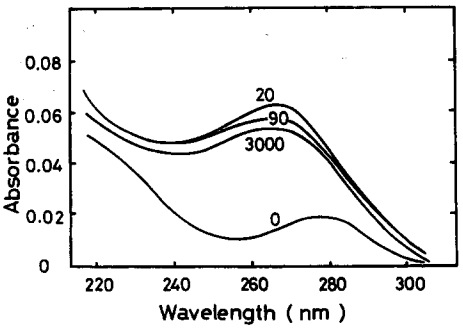


Fig. 7. Spectral changes of reaction products during the reaction with 3,4-dihydroxyphenylacetic acid as a substrate. The concentrations of all ingredients were the same as those described in Fig. 5, in a final volume of 0.15 ml. The reaction was initiated by the addition of the organic substrate at 5°C and stopped by the addition of 0.15 ml of 0.1 M HCl. Numerals in the figure represent the incubation time in seconds. The reaction products were extracted with ethyl acetate and evaporated to dryness at 0°C. The dried residue was taken up in 1.0 ml of ethyl acetate and then 0.2 ml of the resulting sample was subjected to thin-layer chromatography. The remaining sample was evaporated again and dissolved in 2.5 ml of water for measurement of the absorption spectrum. The blank solution was obtained by the similar experiment except for the omission of the organic substrate in the incubation.

shown in Fig. 5 was estimated to be approx. 0.188 mM from the molar absorption differences between the enzyme and the oxygenated intermediate, indicating that more than 90% of the organic substrate added to the reaction mixture participated in the formation of the oxygenated intermediate. The major component extracted from the reaction mixture at 20 s was identified as the end-product by spectrophotometric analysis (Fig. 7) and thin-layer chromatography, confirming that the oxygenated intermediate existed as a species indistinguishable from a complex of reaction end-product and the enzyme.

Conclusions

Since a new spectral species of protocatechuate 3,4-dioxygenase which was an obligatory reaction intermediate was observed during the steady state of the reaction [11,12], it has so far been referred to as 'a ternary complex' (ESO_2) of oxygen (O_2), organic substrate (S), and the enzyme (E) [17] and the reaction mechanism of the enzyme has been proposed as follows [11,17]:

$$E \overset{S}{\rightleftharpoons} ES \overset{O_2}{\rightleftharpoons} ESO_2 \rightarrow E + P$$

The organic substrate combines with the enzyme first to form a binary complex of the enzyme and substrate (ES). Subsequently, the binary complex reacts with oxygen to form a ternary complex of oxygen, substrate, and the enzyme which then degrades to an end-product (P) and the enzyme.

However, the nature of the oxygenated intermediate was still left unsolved and the elucidation of the basic characteristics of which was expected to open the way to a better understanding of the mechanism of oxygenase-catalyzed reactions. The fact that the reaction end-product was quantitatively recovered from the oxygenated intermediate suggests that the oxygenated intermediate

was not a simple complex of oxygen, substrate and the enzyme, i.e., a ternary complex, but species rather close to a binary complex of end-product and the enzyme. The enzymatic reaction, therefore, may proceed via the scheme described as follows:

$$E \overset{S}{\rightleftharpoons} ES \overset{O_2}{\rightleftharpoons} (ESO_2) \rightarrow EP \rightarrow E + P$$

As soon as a ternary complex is formed, the substrate may be rapidly converted to the end-product to form a binary complex of the end-product and the enzyme (EP). Subsequently, the binary complex may liberate the end-product via some conformational changes to form the native enzyme.

Recently, Que et al. investigated the state of the iron and its ligand environment in the enzyme involved in the oxygenated intermediate by EPR and Mössbauer spectroscopy [18,19]. Our experimental result will be helpful for a discussion of their observation.

Involvement of an oxygenated ternary complex in oxygenase reactions has been demonstrated with tryptophan 2,3-dioxygenase [20,21], cytochrome *P*-450 [22,23] and lysine monooxygenase [24]. The study described in this paper indicates that the oxygenated intermediate observed during the reaction of protocatechuate 3,4-dioxygenase is a species close to a complex of end-product and the enzyme, although the possibility that the oxygenated intermediate is a species of a very transient activated state and non-enzymatically yields the end-product during our experimental procedures still remains to be denied.

Acknowledgements

This work has been supported in part by a grant-in-aid for Scientific Research from the Ministry of Education, Science and Culture of Japan, by grant from the Byotai Taisha Research Foundation for 1976, Japan Securities Scholarship for 1976 and by a Naito Research Grant for 1975. The authors would like to express their thanks to Joan Saito for her critical reading of this manuscript.

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