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Naphthalene Metabolism by Pseudomonads: Purification and Properties of 1,2-Dihydroxynaphthalene Oxygenase

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1,2-Dihydroxynaphthalene oxygenase was purified from *Pseudomonas putida* NCIB 9816 grown on naphthalene as the sole source of carbon and energy. The enzyme had a subunit molecular weight of 19,000 and in a medium containing phosphate buffer, 1 mM mercaptoethanol, and 10% (vol/vol) ethanol had a native molecular weight greater than 275,000. The enzyme required Fe^{2+} for activity. It was inactivated slowly on standing, and inactivation was accelerated by dilution with aerated buffers and by H_2O_2. Bathophenanthroline sulfonate, *o*-phenanthroline, 8-hydroxyquinoline, and 2,2'-dipyridyl also inhibited the enzyme. The inactive enzyme was reactivated by anaerobic incubation with ferrous sulfate and ferrous ammonium sulfate. Thiol reagents and acetone, ethanol, or glycerol decreased the rate of loss of activity. The enzyme was most active with 1,2-dihydroxynaphthalene, for which the K_m was 2.8×10^{-4} M. 3-Methyl- and 4-methylcatechols were oxidized at 3 and 1.5%, respectively, of the rate of 1,2-dihydroxynaphthalene, and the K_m for 3-methylcatechol was 1.5×10^{-4} M. Purified 1,2-dihydroxynaphthalene oxygenase catalyzed the oxidation of 1,2-dihydroxynaphthalene, leading to the appearance of 2-hydroxychromene-2-carboxylic acid, but 3-methylcatechol was oxidized by this enzyme to 2-hydroxy-6-oxoheptadienoic acid. Thus, a product structurally analogous to 2-hydroxychromene-2-carboxylic acid was not observed when 3-methylcatechol was oxidized. This may indicate that 2-hydroxychromene-2-carboxylic acid results from cyclization of a ring fission product before release from the enzyme.

1,2-Dihydroxynaphthalene is an intermediate in the metabolism of naphthalene by pseudomonads, and it is oxidized by oxygen in a reaction catalyzed by 1,2-dihydroxynaphthalene oxygenase (7). The enzyme has previously been distinguished from catechol-2,3-dioxygenase by virtue of the greater stability of the latter at 50°C (5) and by a difference in the control of induction (17), but recently we have also shown that the reaction product is the unexpected compound 2-hydroxychromene-2-carboxylic acid (2). This report describes the properties of the enzyme purified from extracts of *P. putida* NCIB 9816 grown on naphthalene and compares the products of the oxidation of 1,2-dihydroxynaphthalene and 3-methylcatechol catalyzed by the enzyme.

MATERIALS AND METHODS

Materials. 2,3-Dihydroxynaphthalene, 3-methylcatechol, 4-methylcatechol, and 2,7-dihydroxynaphthalene were purchased from Aldrich Chemical Co., Inc., Milwaukee, Wis. Pyrocatechol, *o*-phenanthroline, and 3,4-dihydroxybenzoic acid were purchased from J. T. Baker Chemical Co., Phillipsburg, N.J. Bathophenanthroline sulfonate (4,7-diphenyl-1,10-phenanthroline sulfonate), bathocuproine disulfonate (2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline disulfonate), disodium diethydithiocarbamate, 2,2'-dipyridyl, and DEAE-cellulose were purchased from Sigma Chemical Co., St. Louis, Mo. 1,2-Naphthoquinone was purchased from Eastman Kodak Co., Rochester, N.Y. Whenever necessary, commercial products were purified by recrystallization. 1,2-Dihydroxynaphthalene was made by the method of Corner and Young (6) and purified by vacuum sublimation. Sepharose 6B was purchased from Pharmacia, Uppsala, Sweden. All other chemicals were purchased locally and were of analytical grade.

Culture of NCIB 9816. Cultures were grown at 25°C in the mineral medium described previously (18), using naphthalene (2 g/liter) as the sole source of carbon and energy. Four cultures (400 ml each) in 1-liter flasks were grown overnight in a model G76 water bath shaker (New Brunswick Scientific Co., New Brunswick, N.J.) and used to inoculate 10 liters of medium in a fermentor (Microferm, New Brunswick Scientific Co.). The aeration rate was 12 liters/min. Cells were harvested in the late exponential phase of growth (after 10 to 12 h) by centrifugation at 12,000 × *g* for 10 min. They were suspended in 20 mM KH_2PO_4-NaOH buffer (pH 7.2) and centrifuged again. If they were not used immediately, the packed cells were stored at −10°C.

Determination of 1,2-dihydroxynaphthalene oxygenase. Preparations of the enzyme were always reactivated before measurement of their activity. To

a solution of the enzyme in the phosphate medium (pH 6.2, see below) was added a solution of ferrous sulfate (prepared in O_2-free water under a blanket of N_2) to a final concentration of 0.5 mM, and the solution was degassed and stored in an evacuated desiccator for 40 to 60 min. The resulting solution was then used for the determination of enzymatic activity and protein concentration. Reactivated enzyme (5 to 50 μl) was added to 0.1 M acetic acid–NaOH buffer (pH 5.5) to give a final volume of 1.8 ml, and the reaction was started by the addition of 0.5 μmol of 1,2-dihydroxynaphthalene in 10 μl of tetrahydrofuran. The rate of oxygen uptake, initially linear, was measured polarographically at 25°C and recorded on a chart recorder operating at a chart speed of 5 in./min (ca. 12.75 cm/min). Measured rates were corrected for the nonenzymatic rate measured in the presence of heat-inactivated enzyme. The correction was less than 5% of the maximum rates measured. One unit of enzyme is defined as that amount which catalyzes the consumption of 1 μmol of O_2 per min, and specific activity is defined as enzyme units per milligram of protein. Protein was determined by the method of Lowry et al. (13), using bovine serum albumin as a standard. The initial rate of the enzymatic reaction was directly proportional to the concentration of added enzyme in the range of 0 to 2 U per 1.8 ml of reaction mixture, and in this respect the method differs from another polarographic one reported previously (20). High concentrations of Cl^- catalyze the autoxidation of Fe^{2+} and interfere with the determination of the enzyme.

Purification of 1,2-dihydroxynaphthalene oxygenase. Preparations were maintained between 0 and 5°C during purification. Cells were suspended (0.2 g [wet weight] per ml) in 20 mM KH_2PO_4-NaOH buffer (pH 6.2) containing 1 mM 2-mercaptoethanol and 10% ethanol by volume. This mixture will be referred to as phosphate medium, with an additional specification of pH when this was changed. Cells were disrupted with a Sonifier Cell Disrupter (model W185, Heat Systems Ultrasonics, Inc., Plainview, N.Y.), and unbroken cells and debris were removed by centrifugation at 33,000 × g_{av} for 30 min. The supernatant solution (fraction I, Table 1) was centrifuged at 105,000 × g_{av} for 90 min, and the supernatant (II) was removed. Fraction II was centrifuged at 164,000 × g_{av} for 8 h, and the sediment was dissolved in phosphate medium (III). Protamine sulfate (0.1 volume of a 2% [wt/vol] solution) was added, and the resulting precipitate was removed by centrifugation at 33,000 × g_{av} for 30 min. The clear supernatant (fraction IV) was applied to a column (24 cm by 4 cm in diameter) of DEAE-cellulose equilibrated with phosphate medium, and the column was washed with 400 ml of the same buffer. A linear gradient of Na_2SO_4 in phosphate medium was then applied, in which the concentration rose from 0 to 0.25 M in a volume of 1 liter. Fractions which eluted in the concentration range 0.1 to 0.15 M Na_2SO_4 and contained activity were combined (V). Fraction V was dialyzed against phosphate medium (pH 7.2) before fractionation with ammonium sulfate. (Fractionation at pH 6.2 was accompanied by substantial loss of activity.) The dialyzed solution was brought to 35% saturation with prechilled saturated solution of $(NH_4)_2SO_4$ at pH 8.0. After stirring for 15 min, the precipitate was separated by centrifugation (33,000 × g_{av} for 10 min). More $(NH_4)_2SO_4$ was added to give 45% saturation, and after stirring for 15 min, the precipitate was collected by centrifugation. To the clear superantant solution more saturated $(NH_2)_4SO_4$ was added to give 80% saturation. The precipitated protein was collected by centrifugation, dissolved in phosphate medium, and dialyzed against the same medium for 8 h (VI). The increase in total activity at stages III and V was reproducible and may have been due to separation from an inhibitor.

Fraction VI was refractionated on a column (10 cm by 2.5 cm in diameter) of DEAE-cellulose equilibrated with phosphate medium. The column was washed with the same medium (100 ml) and then with a linear gradient of Na_2SO_4 (0 to 0.2 M in 1 liter of phosphate medium). The enzyme was eluted as a single peak in the concentration range 0.1 to 0.14 M Na_2SO_4 (VIII). This material was dialyzed against phosphate medium (pH 7.2) and fractionated with ammonium sulfate as before to give, after dialysis with phosphate medium, fraction VIII.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The methods of Laemmli (12) and Weber and Osborn (19) were used for electrophoresis. Protein solutions were prepared in a sample buffer containing 10% (vol/vol) glycerol, 5% (vol/vol) 2-mercaptoethanol, and 3% (wt/vol) sodium dodecyl sulfate, and the solution was heated for 3 min in a boiling-water bath. Proteins were allowed to traverse the spacer gel at a constant current of 1 mA per gel, and then the current was increased to 2 mA per gel. Gels contained 7.5% polyacrylamide. Electrophoresis was continued at room temperature until the bromophenol blue marker was 1 to 2 cm from the lower end of the

TABLE 1. *Purification of 1,2-dihydroxynaphthalene oxygenase*

Fraction[a]	Total vol (ml)	Total protein (mg)	Total activity (U)	Sp. act. (U/mg)
I 33,000 × *g* supernatant	630	8,100	31,145	3.0
II 105,000 × *g* supernatant	568	6,109	30,193	4.0
III 164,000 × *g* sediment	125	3,715	37,466	10.1
IV Protamine sulfate treated	129	3,176	34,195	10.8
V DEAE-cellulose	385	694	51,205	74.0
VI Ammonium sulfate precipitation	32	603	32,838	54.5
VII DEAE-cellulose	175	311	24,115	76.0
VIII Ammonium sulfate precipitation	12	212	16,224	75.0

[a] Fractions were obtained and assayed as described in the text.

gel. Gels were stained for 8 h in a solution containing 0.1% (wt/vol) Coomassie brilliant blue R, 10% (vol/vol) acetic acid, and 25% (vol/vol) isopropanol. Excess stain was removed electrophoretically.

RESULTS AND DISCUSSION

1,2-Dihydroxynaphthalene oxygenase was fairly stable in crude extracts, which lost about 50% of their activity after 5 days at 4°C. Freezing and thawing caused a negligible loss of activity, but a rapid loss did occur upon dilution or dialysis with aerated buffers or upon treatment with H_2O_2. The loss caused by aerated buffers could be reduced by incorporating a thiol reagent and an organic solvent into the buffer. In the absence of a thiol, ethanol (10% vol/vol) in phosphate buffer (pH 7.2) gave better protection than acetone or glycerol at the same concentration. In the absence of an organic solvent, dithiothreitol, 2-mercaptoethanol, and glutathione each offered better protection than cysteine. Consequently, 10% (vol/vol) ethanol and 1 mM of 2-mercaptoethanol were included in all buffers used in the purification.

The inactive enzyme could be reactivated by anaerobic incubation with a number of reagents, but ferrous sulfate (or ferrous ammonium sulfate) was particularly effective (Table 2). Under aerobic conditions, ferrous sulfate gave only 30% of the activity obtained anaerobically. Reactivation of 1,2-dihydroxynaphthalene oxygenase with ferrous ions has been noted previously (7). Care must be taken to limit the concentration of ferrous ions used to reactivate the enzyme, for the carry over of Fe^{2+} into the assay medium enhances the nonenzymatic oxidation of 1,2-dihydroxynaphthalene. The effect was negligible under the conditions used in this work. Other metal ions (Fe^{3+}, Co^{2+}, Ca^{2+}, Cu^{2+}, Hg^{2+}, Ni^{2+}, Zn^{2+}, Mg^{2+}) could not replace Fe^{2+}, and Hg^{2+}, Cu^{2+}, Co^{2+}, and Fe^{3+} were inhibitory.

The role of ferrous ions in the active enzyme was confirmed by the effect of chelating agents. Those which bind Fe^{2+} most effectively (8-hydroxyquinoline and *o*-phenanthroline) were better inhibitors. Hg^{2+} and *p*-chlormercuribenzoate were particularly inhibitory, which suggests that an enzymatic thiol group also plays a role in the reaction and that the stabilization and stimulatory effect of 2-mercaptoethanol may be due to the maintenance of an enzyme thiol group as much as to the reduction of Fe^{3+} or to the elimination of dissolved oxygen.

The recovery and specific activity of enzyme obtained at each stage of the purification are given in Table 1. Fraction VI was substantially homogeneous on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and fractions VII and VIII showed the presence of only a single subunit with molecular weight 19,000. Gel filtration at 5°C in phosphate medium through Sepharose 6B gave a single symmetrical peak which eluted before xanthine oxidase did. The enzyme therefore had a molecular weight greater than 275,000. The association of subunits may, however, be relatively loose under the conditions of the isolation which were chosen to stablize enzymatic activity. Thus, electrophoresis of the native enzyme in 7.5% polyacrylamide always showed polydispersity. The molecular form of the enzyme in the bacterial cell and during the determination of enzyme activity is therefore uncertain.

TABLE 2. *Reactivation of 1,2-dihydroxynaphthalene oxygenase*[a]

Reagent	Concn (mM) during incubation	Activity (U/ml)
None		9
$FeSO_4$	2.6	129
$FeSO_4$[b]	2.6	40
$Fe(NH_4)_2(SO_4)_2$	2.5	129
$NaBH_4$	3.6	51
Glutathione	3.6	11
2-Mercaptoethanol	9.0	20
Ascorbic acid	3.6	13

[a] An enzyme preparation inactivated by aging was incubated anaerobically (except where stated) with the reagent for 60 min, and the activity was then determined by the standard method. The original preparation had an activity of 135 U/ml (75 U/mg of protein).

[b] Incubated aerobically.

The most highly purified preparation of 1,2-dihydroxynaphthalene oxygenase had an absorption maximum of 280 nm but were colorless in the visible region. In this respect, it is like two other Fe^{2+}-requiring enzymes which catalyze oxidation adjacent to a pair of phenolic hydroxyl groups, catechol-2,3-dioxygenase (15) and 3,4-dihydroxyphenylacetate-2,3-oxygenase (11), and it differs from the red-colored Fe^{3+}-containing catechol-1,2-dioxygenase (10) and protocatechuate-3,4-oxygenase (9), both of which catalyze oxidation between a pair of hydroxyl groups.

The purified enzyme was most active with 1,2-dihydroxynaphthalene, but it also catalyzed the oxidation of 3-methyl- and 4-methylcatechol (Table 3). This activity was not due to a contaminating catechol-2,3-dioxygenase, for the purified enzyme showed no activity with catechol. Furthermore, the activity with 3-methylcatechol fell proportionately to that with 1,2-dihydroxynaphthalene on heating at 50°C. The poorer substrates and other 1,2-dihydric phenols also inhibited the activity of the enzyme with 1,2-dihydroxynaphthalene (Table 3). 3-Methyl- and 4-methyl-catechol were competitive inhibitors

TABLE 3. *Catalytic activity of 1,2-dihydroxynaphthalene oxygenase with various dihydric and trihydric phenols and inhibition by these compounds of the oxidation of 1,2-dihydroxynaphthalene*

Compound	Activity[a] (%)	Inhibition (%)
1,2-Dihydroxynaphthalene	100	
3-Methylcatechol	5	76
4-Methylcatechol	2	36
Pyrocatechol	0	4
2,3-Dihydroxynaphthalene	0	44
3,4-Dihydroxybenzoic acid	0	20
1,2,3-Trihydroxybenzene	0	76
2,7-Dihydroxynaphthalene	0	64

[a] Activity was determined using 0.5 μmol of each compound in the standard method. To measure the rate in the presence of an inhibitor, the latter was added to the reaction mixture immediately before 1,2-dihydroxynaphthalene.

with K_i, values not significantly different from their apparent K_m values. The nature of the inhibition by other components was not investigated.

The optimum pH for the reaction with both 1,2-dihydroxynaphthalene and 3-methylcatechol appeared to be 6.5, but the significance of this value is uncertain because at pH values above 6.5 the nonenzymatic oxidation to 1,2-naphthoquinone, an inhibitor of the enzyme, occurs very rapidly (16). To minimize this effect, the enzyme was routinely assayed at pH 5.5, and this and the addition of a small volume of tetrahydrofuran were important factors contributing to the linear relationship between reaction rate and enzyme concentration (cf. [20]).

The apparent K_m for 1,2-dihydroxynaphthalene was 2.8×10^{-4} M under the conditions of the standard assay, but high concentrations of substrate were inhibitory. Consequently, the measurement of enzymatic activity was made at the prescribed concentration. The apparent K_m for 3-methylcatechol was 0.15 mM and that for oxygen determined by the method of Ferenci (8) was 0.27 mM.

It was shown previously with crude bacterial extracts that the product of the enzymatic oxidation of 1,2-dihydroxynaphthalene (I, Fig. 1) was 2-hydroxychromene-2-carboxylic acid (IV) (2). These observations have now been repeated with the purified enzyme at pH 5.5 and at pH 6.5 with the same results as obtained previously. At higher pH values, the very rapid nonenzymatic oxidation to 1,2-naphthoquinone masks the formation of the product of the enzymatic reaction. These results with the purified enzyme confirm that it is a dioxygenase and not a mixed-function oxidase, even though the overall reaction leads to the insertion of an oxygen molecule in a ring (2).

In an attempt to throw light on the mechanism of the reaction, we also studied the oxidation of 3-methylcatechol catalyzed by 1,2-dihydroxynaphthalene oxygenase and by purified catechol-2,3-dioxygenase (EC 1.13.11.2) obtained from *Pseudomonas* sp. P_G (1) by the method of Nozaki et al. (14). Oxidation with sufficient catechol-2,3-dioxygenase can be completed within 30 s at pH 7.5, and the product (Fig. 2) is relatively stable, showing only a slow decline in absorbance. The absorption spectrum can be changed reversibly by changing the pH, and these changes are consistent with the structure VII (absorbance maximum at 320 nm, ϵ 14.5 mM^{-1} cm^{-1}) ionizing to IX (absorbance maximum at 390 nm, ϵ 39.6 mM^{-1} cm^{-1}) with pK_a 7.6 (Fig. 3). The extinction coefficient at 390 nm is higher than that reported previously (3). These authors reported an extinction coefficient at pH 2.5, which is higher than an observed value at pH 5. We have noted that there is a further reversible absorption increase below pH 4. The chemistry of the oxidation product of 3-methylcatechol has not been described fully. The isolation and identification of a product sterically different from VII have been described, and it has been suggested that isomerization about a double bond occurs during extraction of the product from acid solution (4). Although reversible, pH-dependent spectral changes by the oxidation product have been observed previously (3), the basis of these has not been determined. We suggest that VII ionizes with a pK_a of 7.6 to a resonance-stabilized dianion IX. The configuration of carbon–carbon double bonds in VII would be lost in IX, and reversion to VII could conceivably give a mixture of *cis* and *trans* isomers. This isomerization would probably be rapid, even when the product is formed at a pH which favors the monoanion. Consequently, the

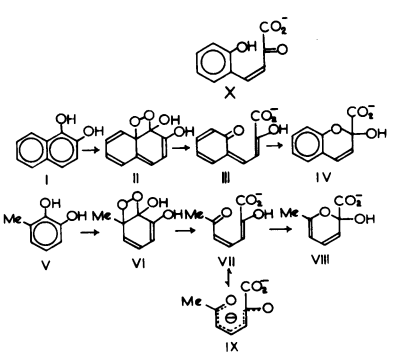

FIG. 1. *Mechanisms for the oxidation of 1,2-dihydroxynaphthalene and 3-methylcatechol catalyzed by 1,2-dihydroxynaphthalene oxygenase. For details see text.*

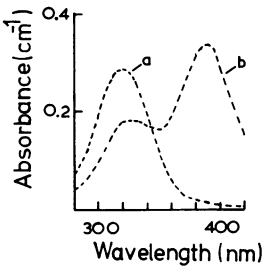

FIG. 2. *Absorption spectrum of the enzymatic oxidation product of 3-methylcatechol prepared at pH 5.5 and 7.5. 3-Methylcatechol (0.019 mM) was oxidized at pH 5.5 (curve a) and at pH 7.5 (curve b). The 1,2-dihydroxynaphthalene oxygenase and catechol-2,3-dioxygenase had specific activities of 70 U/mg of protein and 105 U/mg of protein (measured as in reference 14), respectively. Both reference and sample cuvettes contained buffer and enzyme, and 3-methylcatechol was added to the sample cuvette after it was established that there was no absorption difference between the sample and reference cuvettes. There was no significant difference between the spectra obtained with the two enzymes.*

absorption spectrum which we have associated with VII (curve a, Fig. 2) may be the spectrum of a mixture of isomers.

The oxidation of 3-methylcatechol catalyzed by 1,2-dihydroxynaphthalene oxygenase proceeds much more slowly, and we have been able to complete the reaction only after a period of about 3 min. During the course of the oxidation, at either pH 5.5 or 7.5, the ratio of the absorbance values at 390 and 320 nm was always the same as that of the product of the reaction catalyzed at the same pH by catechol-2,3-dioxygenase, and when the reaction was complete a change in pH produced the same spectral changes. We found no evidence for the presence of any species other than the starting catechol (V) and products VII and IX. If VIII is formed at all, it must have a short half-life in water compared with the relatively short period in which we observed product formation.

Because 3-methylcatechol appears to undergo identical oxidations when catalyzed by catechol-2,3-dioxygenase and by 1,2-dihydroxynaphthalene oxygenase, and because we assume the oxidations of 3-methylcatechol and of 1,2-dihydroxynaphthalene when catalyzed by 1,2-dihydroxynaphthalene oxygenase have a common mechanism, we have presented these latter reactions as shown in Fig. 1. The ketones III and VII are assumed to arise from hypothetical cyclic peroxides II and VI, respectively. Whereas compound VII, 2-hydroxy-6-oxoheptadienoate, and its further ionized form IX are the stable products of the oxidation of 3-methylcatechol, compound III is written only by analogy with VII, and it has not been identified as an intermediate. Invoking a structure such as III does, however, leads to the further possibilities that in the metabolic pathway, compound X might be derived directly from III, that IV is an artifact produced only under the conditions of the enzymatic reaction in vitro, and that the enzyme-catalyzed interconversion of IV and X reported previously (2) proceeds via III. If III does have a free existence, even at a very low concentration, it is difficult to understand why IV is the only product observed in the enzymatic reaction. The chemical stabilization of III by an electron shift could give IV directly, or, when accompanied by a deprotonation and reprotonation, would give X directly. Since proton exchange at hydroxyl groups is very rapid, it is most unlikely that the chemical formation of IV from III would occur to the exclusion of X. Because we have shown previously (2) that at pH 5.5 measurable concentrations of IV and X exist in chemical equilibrium, we conclude that III does not have a free existence and that the exclusive production of IV is determined by the enzyme on which (or as a consequence of the enzymatic environment in which) III is produced. An alternative hypothesis is that III is actually converted to X by the oxygenase and that IV results from a subsequent ring closure. This can be ruled out because the oxygenase and isomerase activities are distinct and because such a process would

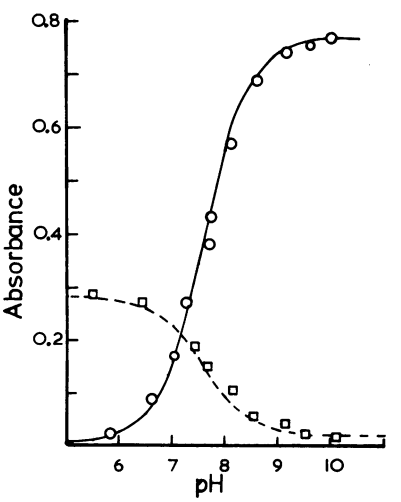

FIG. 3. *The pH dependence of the absorption spectrum of the oxidation product of 3-methylcatechol. The calculated absorbance (○, 390 nm; □, 320 nm) of VII and IX (Fig. 1) together at a total concentration of 0.0194 mM (for IX, ϵ = 39.6 and 1.1 mM^{-1} cm^{-1} at 390 and 320 nm, respectively; for VII, ϵ = 0.3 and 14.5 mM^{-1} cm^{-1} at 390 and 320 nm, respectively, and pK_a 7.6).*

lead to the equilibrium mixture of X and IV and not IV alone (2).

In conclusion, we note that our present proposals suggest that the final product IV results not as originally proposed (2) but as a consequence of ring fission followed by cyclization before release of the product from the enzyme.

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