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Mechanistic Studies on Cyclohexanone Oxygenase†

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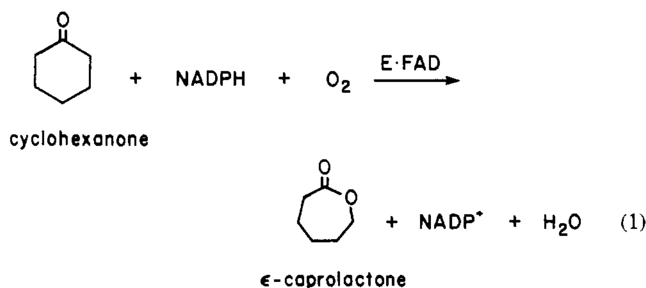
ABSTRACT: The bacterial flavoprotein monooxygenase carries out an oxygen insertion reaction on cyclohexanone, with ring expansion to form the seven-membered cyclic product ε-caprolactone, a transformation quite distinct from the phenol → catechol transformation carried out by the bacterial flavoprotein aromatic hydroxylases. Cyclohexanone oxygenase catalysis involves the four-electron reduction of O_2 at the expense of a two-electron oxidation of NADPH, concomitant with a two-electron oxidation of cyclohexanone to ε-caprolactone. NADPH oxidase activity is fully coupled with oxygen transfer to substrate. Steady-state kinetic assays demonstrate a ter-ter mechanism for this enzyme. Pre-steady-state kinetic assays demonstrate the participation of a 4a-hydroperoxyflavin intermediate during catalysis. In addition to its ketolactonizing activity, cyclohexanone oxygenase carries out S-oxygenation of thiane to thiane 1-oxide, a reaction which represents a nucleophilic displacement by the sulfur upon the terminal oxygen of the hydroperoxide. This is in contrast to cyclohexanone oxygenations where the flavin hydroperoxide acts as a nucleophile. In addition, a stable apoenzyme form is accessible and can be reconstituted with various FAD analogues with up to 100% recovery of enzyme activity. The accumulated results presented here support a Baeyer–Villiger rearrangement mechanism for the enzymatic oxygenation of cyclohexanone.

The assertion has been made (Dagley, 1978) that the microbial world contains at least a few strains that are capable of degrading, under favorable conditions, any one of the innumerable compounds that are biosynthesized by living matter. In addition, a number of these bacteria are capable of utilizing man-made compounds or industrial byproducts, as sole carbon sources. The key strategic catalysts in such processes are the monooxygenases and dioxygenases which introduce an oxygen functionality and set up carbon–carbon bond cleavage steps. The prototypic scheme for metabolism of an aromatic substrate and an aliphatic cyclic substrate is illustrated by the converging pathways for the microbial metabolism of *p*-hydroxybenzoate and cyclohexanol shown in Scheme I; the convergent metabolite is the acyclic β-ketoadipate. A key step early in each branch of this microbial metabolism involves a flavin-dependent monooxygenase: in the one case an aromatic ring hydroxylase, *p*-hydroxybenzoate hydroxylase, and in the other a ketone monooxygenase, cyclohexanone oxygenase. A plethora of kinetic and chemical data regarding the catalytic mechanism of *p*-hydroxybenzoate hydroxylase (Entsch et al., 1974, 1976a, 1980; Husain & Massey, 1979) has been accumulated and makes this enzyme the paradigm for bacterial flavoprotein monooxygenases which utilize phenolic substrates.

Cyclohexanone oxygenase (EC 1.14.13), first purified by Trudgill and colleagues (Donoghue et al., 1976), is one of a small class of bacterial flavoprotein monooxygenases which operates on a distinct structural type of substrate in carrying out a ketone → lactone conversion; in contrast, *p*-hydroxybenzoate hydroxylase performs a phenol → catechol conversion. The stoichiometry of the cyclohexanone oxygenase reaction is shown in eq 1; it involves the four-electron reductive



splitting of O_2 at the expense of a two-electron oxidation of NADPH, concomitant with oxygen transfer to cyclohexanone and ring expansion to form the seven-membered cyclic product ε-caprolactone.

In an effort to elucidate the mechanism by which the oxygen insertion reaction on cyclohexanone is effected, this paper presents information about the reaction mechanism of cyclohexanone oxygenase and includes steady-state kinetics, product analysis, pre-steady-state kinetics, and apoenzyme reconstitution assays with flavin analogues of FAD. The accumulated results support a Baeyer–Villiger rearrangement mechanism for the enzymatic oxygenation of cyclohexanone. A Baeyer–Villiger rearrangement is defined as an oxygen insertion reaction resulting from the treatment of a ketone with a peracid

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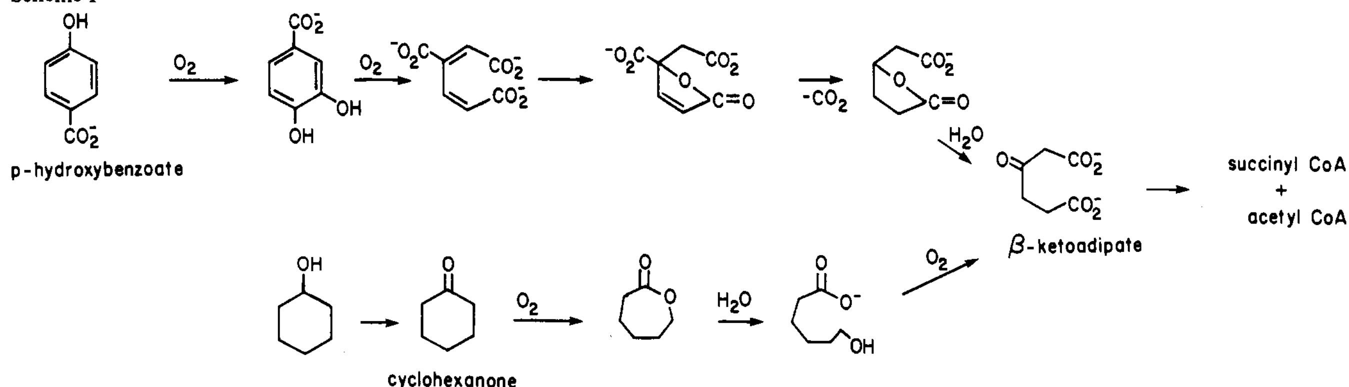
Scheme I



or other peroxy compound (March, 1968).

Materials and Methods

Materials

A culture of *Acinetobacter* NCIB 9871 was a generous gift from Dr. P. W. Trudgill, at the University College of Wales, Aberystwyth, United Kingdom. [1-^{14}C]Cyclohexanone (8.4 or 15 mCi/mmol) and sodium boro[^{3}H]hydride (>100 mCi/mmol) were obtained from New England Nuclear. Sodium boro[^{2}H]hydride and [2,2,6,6-$^2H_4$]cyclohexanone (98% atom D) were purchased from Merck and Co., Inc.

The various ketone substrates and lactone products were purchased from either Fisher Scientific Co. or Aldrich. All other chemicals and biochemicals were purchased from Sigma or Boehringer Mannheim. All substrate solutions were prepared fresh daily.

The flavin analogues of FAD were prepared in collaboration with Dr. R. W. Spencer and Dr. D. Light of the laboratory at M.I.T. (Walsh et al., 1978; Spencer et al., 1976; Light et al., 1980).

Methods

Culture Medium and Growth of Bacteria. Stock cultures of *Acinetobacter* NCIB 9871 were grown on nutrient agar at 30 °C and stored at 4 °C and subcultured at monthly intervals. Large batches of cells (100 g in 25 L) were grown essentially according to Donoghue et al. (1976) with the following modifications. Cells were grown at 30 °C in medium containing (g/L) the following: KH_2PO_4, 2.0; Na_2HPO_4, 4.0; $(NH_4)_2SO_4$, 3.0; yeast extract, 0.2; this solution was brought to pH 7.5 and sterilized by autoclaving. At the time of inoculation, concentrated solutions of each of the following compounds were sterilized by membrane filtration and then added to the phosphate salts to give the indicated final concentration (g/L): $MgSO_4 \cdot 7H_2O$, 0.5; $CaCl_2 \cdot H_2O$, 0.1; $FeSO_4 \cdot 7H_2O$, 0.01; cyclohexanol, 0.1 or 1.0 (as specified below). The sequence of transfers for large batches of cells was as follows. Mineral salts–cyclohexanol (0.1 g/L) medium, 50 mL in a 250-mL Erlenmeyer flask, was inoculated from an agar stab, and the culture was grown for 12 h at 30 °C with gentle shaking. At this time, additional cyclohexanol (1.0 g/L) was introduced to each flask. After an additional 7–8 h growing time, this 50-mL culture was used in toto to inoculate 1 L of mineral salts–cyclohexanol (1.0 g/L) medium in a 4-L Erlenmeyer flask. This was shaken for 12 h at 30 °C and then was added to a 25-L fermentor (New Brunswick Scientific Co., New Brunswick, NJ) containing 24 L of mineral salts–cyclohexanol (1.0 g/L) medium. The cells were grown at 30 °C with vigorous aeration and stirred at 400 rpm. Cyclohexanol (1.0 g/L) was added at the following intervals: (1) after 10–11 h; (2) 1 h after the first addition; (3) 1 h after the second addition. The cells were harvested, using a Sorvall KSB, Szent-Gyorgi and Blum continuous flow apparatus, immediately following the third addition of cyclohexanol. The cell pellets were washed by resuspension in phosphate buffer (KH_2PO_4, 2 g/L, and Na_2HPO_4, 4 g/L, pH 7.1) followed by centrifugation at 4 °C and 15000*g* for 1 h. The cell paste was either used directly or stored at −20 °C for up to 9 months without loss of enzyme activity. This procedure results in typical yields of 100 g wet weight of cells.

Enzyme Purification. The purification was carried out exactly as described by Donoghue et al. (1976) and involved ammonium sulfate fractionation, a DE-52 (DEAE-cellulose)[1] column, and an Ultrogel ACA-44 (4% agarose + 4% polyacrylamide) column. The final hydroxylapatite column used by Donoghue was eliminated.

Enzyme Assays. Cyclohexanone oxygenase activity was routinely assayed at 30 °C by monitoring NADPH oxidation at λ 340 nm on a Perkin-Elmer 200, 554, or 557 spectrophotometer. A standard assay solution contained 1 mL of 80 mM glycine–NaOH, pH 9.0, 0.16 mM NADPH, 48.5 μM cyclohexanone, and 0.011–0.11 μM cyclohexanone oxygenase (concentration based on flavin, ϵ_{440} = 11.3 mM^{-1} cm^{-1}). Atmospheric oxygen concentration (0.24 mM) was present in all assays unless otherwise specified. Specific activity was expressed as micromoles per minute per milligram of protein.

Pre-Steady-State Assays. Rapid reaction kinetic experiments were performed on an anaerobic stopped-flow spectrophotometer as described in Beaty & Ballou (1981a); the path length of the observation cell was 2 cm, and the dead time was 2.5–3 ms. Experiments were performed to investigate both the reductive half-reaction (reduction of enzyme–flavin by NADPH) and the oxidative half-reaction (reoxidation of the reduced enzyme–flavin complex by O_2). The anaerobic reduction of the enzyme–flavin chromophore was monitored as a decrease in absorbance at λ 440 nm. These assay mixtures contained equal volumes of the following two solutions: (1) 409 μM NADPH and 392 μM $NADP^+$ in 80 mM glycine–NaOH, pH 9.0; (2) 97 μM cyclohexanone and 32 μM cyclohexanone oxygenase in 20 mM KP_i, pH 7.2. After being mixed in the stopped-flow apparatus, the solution had a final pH of 8.7. Other assays were done completely in 20 mM KP_i, pH 7.2, to investigate effects of pH on the reductive half-reaction. All assays were done at 3–5 °C and under strict anaerobisis. Reoxidation of reduced cyclohexanone oxygenase was monitored at several different wavelengths between 320

[1] Abbreviations: GLC-MS, gas-liquid chromatography-mass spectrometry; HPLC, high-pressure liquid chromatography; DEAE-cellulose, diethylaminoethylcellulose; EDTA, ethylenediaminetetraacetate; DTT, dithiothreitol; Tris, tris(hydroxymethyl)aminomethane; $NaDodSO_4$, sodium dodecyl sulfate.

and 650 nm with 0.06–0.60 mM oxygen. Assays were done at both pH 8.7 (equal volumes of 80 mM glycine, pH 9.0, and 20 mM KP_i, pH 7.1) and pH 7.2 (20 mM KP_i, pH 7.2, only). A typical assay in 200 μL of buffer contained 14 mM EDTA, 0.26 μM 5-deaza-FAD, 0.009–0.016 mM cyclohexanone oxygenase (in the redued form), 0.19 mM $NADP^+$, and 48.5 μM cyclohexanone. For maintenance of anaerobic conditions, 0.018 mM protocatechuic acid and ~30 μM protocatechuic dioxygenase (prepared from *Pseudomonas putida* by C. Bull at the University of Michigan) were added to the assays. In preparation for reaction with oxygen, the enzyme in these experiments was reduced photochemically in buffered solution in a nitrogen atmosphere by irradiation with a sun gun at 80 V, 4 cm from the sample for 10–20 min at 3–5 °C in the presence of 5-deaza-FAD and EDTA (Massey & Hemmerich, 1978), or by stoichiometric titration with NADPH (in these assays, 5-deaza-FAD and EDTA were not present). The analysis of absorption changes during reactions to obtain spectra of intermediates was performed as described in Entsch et al. (1976a). The determination of rate constants was complicated by the multiphasic (>2 phases) reaction data; in time intervals where the reaction was biphasic, the data were analyzed as described in Henkens & Turner (1973).

HPLC Separation of Cyclohexanone and ε-Caprolactone. An analytical HPLC separation of cyclohexanone and ε-caprolactone was accomplished by using a μBondapak C_{18} column (Waters Associates, Inc., 0.39 × 30 cm) with 14% acetonitrile–water, at a flow rate of 1.0 mL/min. The HPLC system used in these experiments was a Waters Associates, Inc., instrument equipped with a refractive index detector. The retention times of the ketone and lactone were established by using authentic samples of each and are 12.4 and 7.8 min, respectively, as determined by the refractive index. An independent hydroxamate assay (Lipmann & Tuttle, 1945) was used to identify the lactone isolated by HPLC in a nonradioactive experiment. Solution A was 1 mL each of 4 N NH_4OH and 4 N NaOH (pH 7–8). Solution B was 1 mL each of 5% $FeCl_3$ in 0.1 N HCl, 12% trichloroacetic acid, and 3 N HCl. Solution A (100 μL) and a lactone sample (300–2000 nmol of ε-caprolactone + 80 mM glycine–NaOH, pH 9.0, to give 200 μL total volume) were incubated at room temperature for 10 min. Solution B (300 μL) was added, and the hydroxamate–Fe complex was measured at λ 540 nm, ϵ_{540} = 0.07 mM^{-1} cm^{-1}. This assay could detect a minimum of 300 nmol of lactone.

The assay mixtures used in the quantitation of product (ε-caprolactone) contained exactly the same components as described above for cyclohexanone oxygenase assays except that ~300 nmol of [1-^{14}C]cyclohexanone (1.14 mCi/mmol) was added as substrate. After ~20% of the NADPH had been oxidized (monitored at 340 nm), a 400-μL aliquot was withdrawn (in a temperature-controlled syringe at 30 °C) onto Dowex 1-Cl^- (0.5 × 3 cm) to quench the reaction. The solution was immediately forced through the Dowex 1-Cl^-. Samples of nonradioactive ketone (2 μmol) and lactone (2 μmol) were added to the eluate from the Dowex column as carrier, to enable detection by the refractive index detector. A 200-μL aliquot of this solution was loaded onto the analytical reverse-phase column and eluted with 14% acetonitrile–water, at 1.0 mL/min; 300-μL fractions were collected and counted (1 nmol of ^{14}C = 2000 cpm).

An assay mixture used to test the reactivity of H_2O_2 contained 0.028 μM cyclohexanone oxygenase, 0.19 mM $NADP^+$, 0.18 mM H_2O_2, and 0.27 mM (1.61 mCi/mmol) [1-^{14}C]-cyclohexanone in 1 mL of 80 mM glycine–NaOH, pH 9.0, which was incubated at 30 °C for 5 min. A 400-μL aliquot was then analyzed as above, except that 150 μL of the carrier-containing solution was applied to the reverse-phase column. The cyclohexanone and lactone were separated as described above (1 nmol of ^{14}C ≃ 3000 cpm).

Apoenzyme Preparation. Typically 500 μL of cyclohexanone oxygenase (8.7 mg/mL) was dialyzed against four changes each of 1 L of 2 M KBr, 0.1 M KH_2PO_4, 0.3 mM EDTA, and 1 mM DTT, at pH 7.1 and 4 °C for 48 h. Dialysis was continued for another 24 h in three changes each of 1 L of the same buffer without KBr. The resultant protein was nearly colorless and was assayed for protein (by A_{280}) and activity. A typical reconstitution assay was carried out as follows: 0.5 mL of 80 mM glycine–NaOH, pH 9.0, 0.16 mM NADPH, 0.012 mg of apocyclohexanone oxygenase (concentration was determined by using 1 OD_{280} ≃ 1 mg/mL), and 0.58 μM FAD (or FAD analogue) was incubated at 30 °C for 1 min, and then cyclohexanone (final concentration 0.048 mM) was added. Activity was monitored by NADPH consumption on a Perkin-Elmer 220, 554, or 557 spectrophotometer. Apoenzyme which had been stored at 4 °C for up to 1 month could be fully reconstituted.

Flavin Analogues—Preparation and Purification. The riboflavin level of each flavin analogue was prepared as cited: 5-deazariboflavin (O'Brien et al., 1970); 1-deazariboflavin (Ashton et al., 1977); 9-azariboflavin (Graham et al., 1977); 7-chloro-8-demethylriboflavin (Shunk et al., 1952); 6-methylriboflavin (Berezovskii & Rodionova, 1958). Flavokinase/FAD synthetase from *Brevibacterium ammoniagenes* was used in the syntheses of the various FAD analogues as reported in Spencer et al. (1976). The purification of the various analogues was achieved by HPLC (Light et al., 1980).

Isolation and GLC–MS Analysis of Cyclohexanone and ε-Caprolactone. Incubations with [2,2,6,6-$^2H_4$]cyclohexanone contained 67.6 μmol of ketone, 20 units of glucose-6-phosphate dehdyrogenase, 81.1 μmol of glucose 6-phosphate, 1.0 μmol of NADPH, and 2.0 units of cyclohexanone oxygenase in 3 mL of 0.2 M Tris, pH 8.0. The resulting solution was allowed to sit overnight in a stoppered test tube at ambient temperature. The incubation mixture was then extracted with 0.5 mL of $CHCl_3$. The $CHCl_3$ extract was dried over Na_2SO_4, filtered, and evaporated under a stream of N_2 to 20–30 μL. Samples were prepared in $CHCl_3$ of ε-[2,2,6,6-$^2H_4$]caprolactone [prepared according to Friess (1949)] and [2,2,6,6-$^2H_4$]cyclohexanone for comparison with compounds extracted from the enzymatic incubation in GLC–MS analysis.

A Hewlett-Packard 5990A gas chromatograph–mass spectrometer (70-eV ionization potential) equipped with a 6 ft × $^1/_8$ in. column packed with 3% SE-30 on 80/100 mesh Chrom-Q was used for the analyses described above. The following GLC conditions were used: gas flow, 30 mL/min; oven, 50–250 °C at a rate of 16 °C/min; injection port, 200 °C. The retention times for ketone and lactone were 2–3 and 5–6 min, respectively.

Enzyme Assays with Thiane. Cyclohexanone oxygenase (10 units) was immobilized by condensation copolymerization into a cross-linked polyacrylamide gel according to Pollak et al. (1980). This immobilized enzyme was incubated with immobilized glucose-6-phosphate dehydrogenase (30 units, prepared by the same procedure), 5.8 mM glucose 6-phosphate, 0.20 mM NADPH, and 4.8 mM thiane in 500 mL of 0.1 M Tris, pH 8.3, at room temperature. After 4 days, 0.8 mmol of sulfoxide (thiane 1-oxide) was produced; the immobilized cyclohexanone oxygenase was 30% active (with cyclohexanone) at this point. Attempts were not made to op-

Table I: Purification of Cyclohexanone Oxygenase[a]

step	total protein (mg)	sp act. (μmol min^{-1} mg^{-1})	A_{280}/A_{440}
DE-52 column	236.8	7.09	23
Ultrogel AcA44 column	30.6	11.8	13.6

[a] The data presented here are for an enzyme preparation resulting from an original suspension of 100 g wet weight of *Acinetobacter* NCIB 9871. The procedure is described under Methods.

timize the reaction conditions here. The production of thiane 1-oxide was monitored by GLC on a Perkin-Elmer 3920 (FID) with a 10% Carbowax 20M column, 6 ft, 80–100 mesh. The retention times for the sulfide and sulfoxide were 1.1 and 12.4 min, respectively, under the following conditions: gas flow, 41 mL/min; oven, 180 °C; injection port, 200 °C. An authentic sample of thiane 1-oxide was prepared (Lambert & Keske, 1966) by the oxidation of thiane with H_2O_2 and was found to have a retention time by GLC identical with that of the enzymatically produced oxide. This immobilization technique was used since, at concentrations of sulfide up to 22 mM (necessary to generate enough sulfoxide to be detectable by GLC), soluble cyclohexanone oxygenase is unstable. The immobilized enzyme did not experience this organic solvent toxicity, and, in addition, it was easily recovered from large (500 mL) reaction volumes by centrifugation. Experimentation is continuing to investigate the utility of cyclohexanone oxygenase as a preparative reagent for lactone formation. These experiments were carried out in collaboration with Obsidiana Abril and G. M. Whitesides at M.I.T., Cambridge, MA.

Results

Purification of Cyclohexanone Oxygenase. A typical purification procedure yielded 30.6 mg of protein at a specific activity of 11.8 units/mg of protein from 100 g of cells; the results are presented in Table I. By the criteria of both specific activity and protein–flavin content, our enzyme preparations are ca. 80% pure. The protein, by $NaDodSO_4$ gel electrophoresis (Weber & Osborn, 1969), was 80–90% pure, with one major band [at M_r 56 000, identical with that determined by Donoghue et al. (1976)], and three or four minor bands each comprising less than 5% of the total protein. Only the major protein band from nondenaturing gel electrophoresis of native cyclohexanone oxygenase showed any enzymatic activity.

The turnover number for this cyclohexanone oxygenase as determined by flavin content is 30.3 s^{-1} [compared to the value 23.2 s^{-1} extrapolated from published data (Donoghue et al., 1976)], as measured by NADPH consumption at 340 nm, pH 9.0, and 30 °C. The pH and temperature dependence of turnover is summarized in Table II. Of primary interest in this study of the enzyme is the question of how the oxygenation of cyclohexanone to ε-caprolactone is effected (see eq 1). Before the chemistry of this reaction was analyzed, the sequence of the substrates and products involved in the enzyme reaction was determined.

Coupling of NADPH Oxidation to Oxygen Consumption. Cyclohexanone oxygenase has a fairly broad substrate specificity for cyclic ketones (e.g., cyclobutanone through cyclooctanone, 2-substituted cyclohexanones, and camphor) but will not oxidize aliphatic ketones (e.g., acetone and methyl ethyl ketone) (Donoghue et al., 1976). Under normal assay conditions, cyclohexanone enhances O_2-dependent NADPH consumption rates by a factor of 30, in contrast to the aromatic

Table II: Turnover Numbers for Cyclohexanone Oxygenase

temp (°C)	pH	turnover no. (s^{-1})[a]	[NADP$^+$] (mM)
4	7.0	0.49	0
		0.49	0.21
4	9.0	2.26	0
		3.5	0.21
30	9.0	30.3	0
		30.3	0.27
		23.2[b]	0

[a] These V_{max} values were determined in steady-state assays with cyclohexanone oxygenase and cyclohexanone as substrate. The assay conditions are described under Methods. [b] This is the value reported by Donoghue et al. (1976) and measured at the indicated conditions.

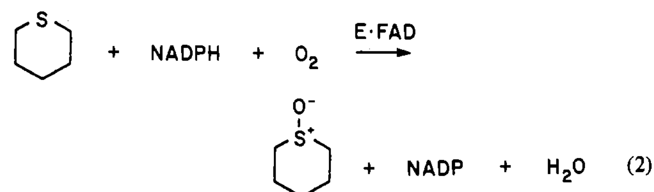
Table III: Michaelis Constants for Substrates and Products

substrate	K_m (μM)	K_D (μM)
NADPH	20	11[a]
cyclohexanone	4	6[b]
O_2	<100	ND[c]
product	**K_I (μM)**	**K_D (μM)**
NADP$^+$	38[d]	ND
ε-caprolactone	none detected	ND

[a] Determined in pre-steady-state assays. [b] Determined by spectrophotometric titration of free oxidized enzyme with cyclohexanone and then recording difference spectra. [c] ND = not determined. [d] Inhibition was observed against NADPH but not against cyclohexanone.

phenolic hydroxylases where the presence of phenolic substrates enhances activity up to 10^4-fold (Howell et al., 1972). The K_m value measured for this enzyme for cyclohexanone and NADPH are 4 and 20 μM, respectively (Table III).

We now find that an alternate substrate is the 6-membered cyclic sulfide thiane (pentamethylene sulfide), which is processed to the product *S*-oxide as shown in eq 2 in a reaction

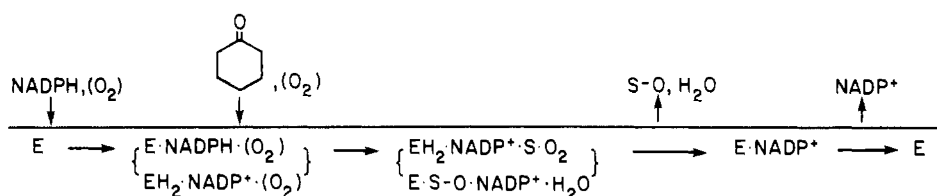


which is fully coupled to NADPH oxidation. The thiane 1-oxide product was identified by gas chromatographic retention time identity with authentic sulfoxide. These incubations were performed with enzyme immobilized as described under Methods to improve stability in long incubations required for accumulation of substantial amounts of product. The V_{max} for sulfoxidation under standard assay conditions was 0.88 of that for cyclohexanone, and the K_m for thiane was 24 μM.

Cyclohexanone oxygenase was found to consume NADPH at the same rate as O_2 uptake (O_2 electrode assay) in the presence of either cyclohexanone or thiane under all assay conditions. In the presence of limiting amounts of substrate, catalase had no effect on O_2 uptake. Final proof that NADPH oxidation was fully coupled to substrate oxygenation was achieved by an assay with [1-^{14}C]cyclohexanone. The unreacted cyclohexanone and the product, ε-caprolactone, were separated by HPLC as described under Methods. Upon the oxidation of 16 ± 1 nmol of NADPH, 17 ± 2 nmol of ^{14}C-labeled lactone were produced.

Steady-State Kinetic Pattern. For establishment of the order of addition of substrates onto the enzyme and the order

Scheme II



of the products released, assays were conducted which yield the following patterns. The steady-state kinetic pattern, generated from varying cyclohexanone concentrations at different fixed NADPH concentrations, is parallel. This indicates that cyclohexanone and NADPH bind to different forms of the enzyme. Consistent with this hypothesis is the finding that the rate of FAD reduction on the enzyme by NADPH is independent of cyclohexanone concentration (see below under pre-steady-state experiments). No dependence of overall rate could be demonstrated with varying oxygen concentration (0.10–0.50 mM) at different fixed concentrations of NADPH; therefore, no kinetic pattern can be generated. It was concluded, then, that the K_m for oxygen must be <10–15 μM—an unusual feature of this enzyme since most other flavoproteins have a K_m for O_2 in the 30–100 μM range (Howell et al., 1972; Neujahr & Gaal, 1973; Takemori et al., 1972; Strickland & Massey, 1973).

The product, $NADP^+$, is a competitive inhibitor (K_I = 38 μM) against NADPH but shows no detectable inhibition against cyclohexanone; the V_{max} is unaffected by $NADP^+$ concentration (see Table II). The other product, ε-caprolactone, does not show any measurable inhibition against either NADPH or cyclohexanone in assays containing 90 μM ε-caprolactone, 30–40 μM NADPH (K_m = 20 μM), and 3–19 μM cyclohexanone (K_m = 4 μM). This is consistent with NADPH and $NADP^+$ binding to the same form of the enzyme, namely, oxidized enzyme (E–FAD); therefore, the binding of cyclohexanone or ε-caprolactone to free enzyme is not kinetically significant during turnover. The binding constants (K_D), which can be measured for cyclohexanone and NADPH, are 6 and 11 μM, respectively; these values are very close to K_m (see Table III). It is possible that cyclohexanone does bind to the enzyme in random order with respect to NADPH but that its binding has no effect upon the reaction with NADPH.

A kinetic mechanism consistent with these data is summarized in Scheme II in shorthand notation (Cleland, 1963). The order of addition of O_2 during turnover could not be determined by the usual methods of varying oxygen concentration at fixed levels of the other two substrates. Addition of oxygen with NADPH to free enzyme might require that a stable, and therefore spectrally visible, flavin–oxygen intermediate be formed in the absence of cyclohexanone. No such intermediate can be observed except under pre-steady-state conditions (see below). In light of this experimental evidence and literature precedents which demonstrate that, without exception, the bacterial flavin-dependent phenolic monooxygenases, which have been studied, add organic substrate before oxygen (Kamin et al., 1978; Husain et al., 1978; Hayaishi, 1974), it seems most likely that oxygen adds to the reduced enzyme–$NADP^+$ complex immediately following cyclohexanone.

Pre-Steady-State Kinetics. In pre-steady-state assays, we were able to detect and identify transient flavin–oxygen intermediates and also to assign some rate constants to specific catalytic steps. The redox reaction catalyzed by cyclohexanone oxygenase was studied in terms of two half-reactions: the reductive half-reaction (eq 3), in which enzyme-bound FAD is reduced by NADPH, and the oxidative half-reaction, in which the reduced flavin is reoxidized by molecular oxygen concomitant with oxygenation of the ketone (eq 4c). We also examined E–$FADH_2$ reoxidation in the absence of both NADP and cyclohexanone (eq 4a) or in the absence of cyclohexanone (eq 4b). In order to investigate each half-reaction separately, we performed single turnover assays with cyclohexanone oxygenase by using anaerobic stop-flow techniques.

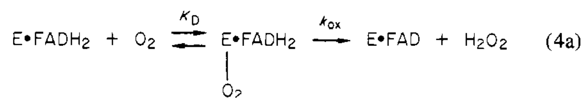
(*A*) *Reductive Half-Reaction.* Pseudo-first-order rate constants for flavin reduction by excess NADPH were determined under strict anaerobic conditions, at pH 7.2 and 8.7 at 4 °C, by monitoring the decrease in absorbance at λ 440 nm due to the enzyme-bound oxidized flavin (eq 3). The

$$\mathrm{E{\cdot}FAD + NADPH \underset{}{\overset{K_D}{\rightleftarrows}} E{\cdot}FAD{\cdot}NADPH \xrightarrow{k_{red}} E{\cdot}FADH_2{\cdot}NADP^+} \quad (3)$$

constants k_{redn} and K_D were determined as described by Strickland et al. (1975). The dissociation constant calculated (at pH 7.2) for the binding of NADPH to cyclohexanone oxygenase is K_D = 11 μM. The rate of flavin reduction at pH 8.7 is k_{red} = 19.4 s^{-1}, and at pH 7.2, it is k_{redn} = 17.5 s^{-1} and is independent of cyclohexanone concentration. Since the ketone substrate does not affect the flavoprotein reduction rate, but yet it enhances turnover by 30-fold, the presence of ketone (or sulfide) must selectively accelerate the oxidation rate of E–$FADH_2$.

(*B*) *Oxidative Half-Reaction.* Cyclohexanone oxygenase was photochemically reduced with light, 5-deazaflavin, and EDTA, or by stoichiometric NADPH titration, under anaerobic conditions, and then mixed with buffer containing oxygen in the stopped-flow apparatus. The reoxidation of the two electron reduced flavin was monitored at a number of different wavelengths between 320 and 650 nm. Assays were conducted at 4 °C in the presence of $NADP^+$ (0.19 mM), at pH 8.7 where the activity is maximal and also at pH 7.2 where the reaction will proceed at a slower rate (to aid in the search for any flavin–oxygen intermediates which might be formed). The oxidative half-reaction was measured in the absence of cyclohexanone as an NADPH oxidase activity (eq 4a and 4b), and in the presence of cyclohexanone (0.097 mM) as an oxygenase activity (eq 4c, the normal turnover cycle).

(*C*) *Oxidase Reaction in the Absence of NADP and Cyclohexanone.* The log plots of the rate data are linear to, greater than, or equal to five half-lives, and when treated as a two-step reaction (eq 4a), proceeding under pseudo-first-



$$\mathrm{E{\bullet}FADH_2 + O_2 \overset{K_D}{\rightleftarrows} E{\bullet}FADH_2{-}O_2 \xrightarrow{k_{ox}} E{\bullet}FAD + H_2O_2} \quad (4a)$$

order conditions, K_D = 2 mM for oxygen and k_{ox} = 4.5 s^{-1}. No 4a-hydroperoxyflavin intermediate could be detected in this reaction without $NADP^+$ present. The spectrum observed in the dead time of the apparatus was that of reduced enzyme. In our catalytic turnover experiments, excess $NADP^+$ is always present; therefore, this oxidase reaction (eq 4a) does not appear to apply in catalysis. Examination of the kinetic mechanism (Scheme II) for this enzyme shows that $NADP^+$ stays bound

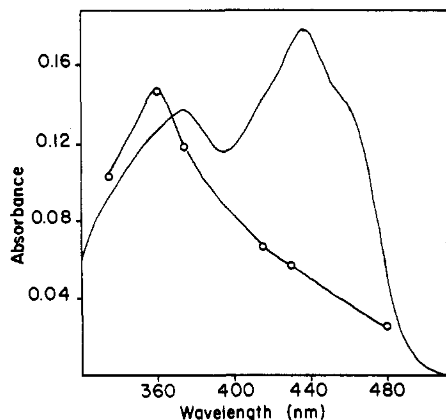


FIGURE 1: Spectrum indicated by the open circles is calculated 10 ms after the initiation of the reaction of reduced cyclohexanone oxygenase (0.014 mM) with oxygen (0.60 mM) at pH 8.7 and 4 °C. The solid line is the spectrum of fully oxidized cyclohexanone oxygenase in solution at the end of the reaction.

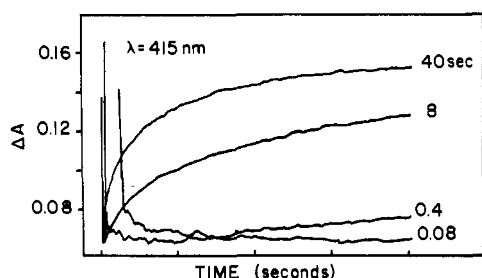
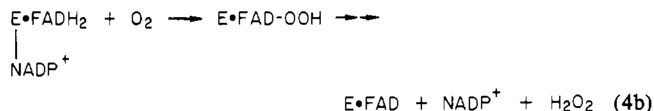


FIGURE 2: Time course of absorption at 415 nm upon reacting oxygen (0.6 mM) with reduced cyclohexanone oxygenase (0.014 mM) in the absence of any ketone substrate. These traces were recorded at pH 7.2 and 4 °C as described under Methods.

to the enzyme and is released in the last step; therefore, $NADP^+$ must be present in assays of the oxidase half-reaction.

(*D*) *Oxidase Reaction in the Absence of Cyclohexanone.* The oxidase reaction described by eq 4b was measured in the absence of cyclohexanone and in the presence of 0.19 mM $NADP^+$ at pH 7.1 and 4 °C by reacting the reduced flavo-



$$\underset{\displaystyle NADP^+}{E{\cdot}FADH_2} + O_2 \rightarrow E{\cdot}FAD\text{-}OOH \rightarrow\rightarrow E{\cdot}FAD + NADP^+ + H_2O_2 \quad (4b)$$

protein in the presence of $NADP^+$ with 0.6 mM oxygen. A flavin–oxygen intermediate, proposed from its electronic spectrum to be a 4a-peroxyflavin [by analogy to Entsch et al. (1976a)] (Figure 1), is formed within the dead time (2–3 ms) of the instrument; therefore, the reaction of the reduced flavin–enzyme–$NADP^+$ complex reacts with O_2 at $k > 1000\ s^{-1}$ (at this concentration of oxygen). This corresponds to a second-order rate constant $\geq 1.7 \times 10^6\ M^{-1}\ s^{-1}$ and is much faster than any other analogous reactions measured with other flavoprotein hydroxylases (Entsch et al., 1976a). Even at 30 $\mu M\ O_2$, the reaction is complete within the dead time. Hence, the true rate constant must be $>3 \times 10^7\ M^{-1}\ s^{-1}$. For comparison, the reaction of free dihydroflavin with oxygen, under similar conditions, is $\sim 50\ M^{-1}\ s^{-1}$. Thus, cyclohexanone oxygenase accelerates this reaction about 1 000 000-fold.

The decay of the 4a-peroxyflavin proceeds at a rate of $k = 25 \pm 2\ s^{-1}$, followed by several slow phases (0.77–0.12 s^{-1}) some of which may involve charge-transfer complexes. Figure 2 demonstrates the complexity of these rate data.

(*E*) *Oxygenase Reaction.* The oxygenation of cyclohexanone (0.097 mM) or of thiane (0.29 mM) was observed

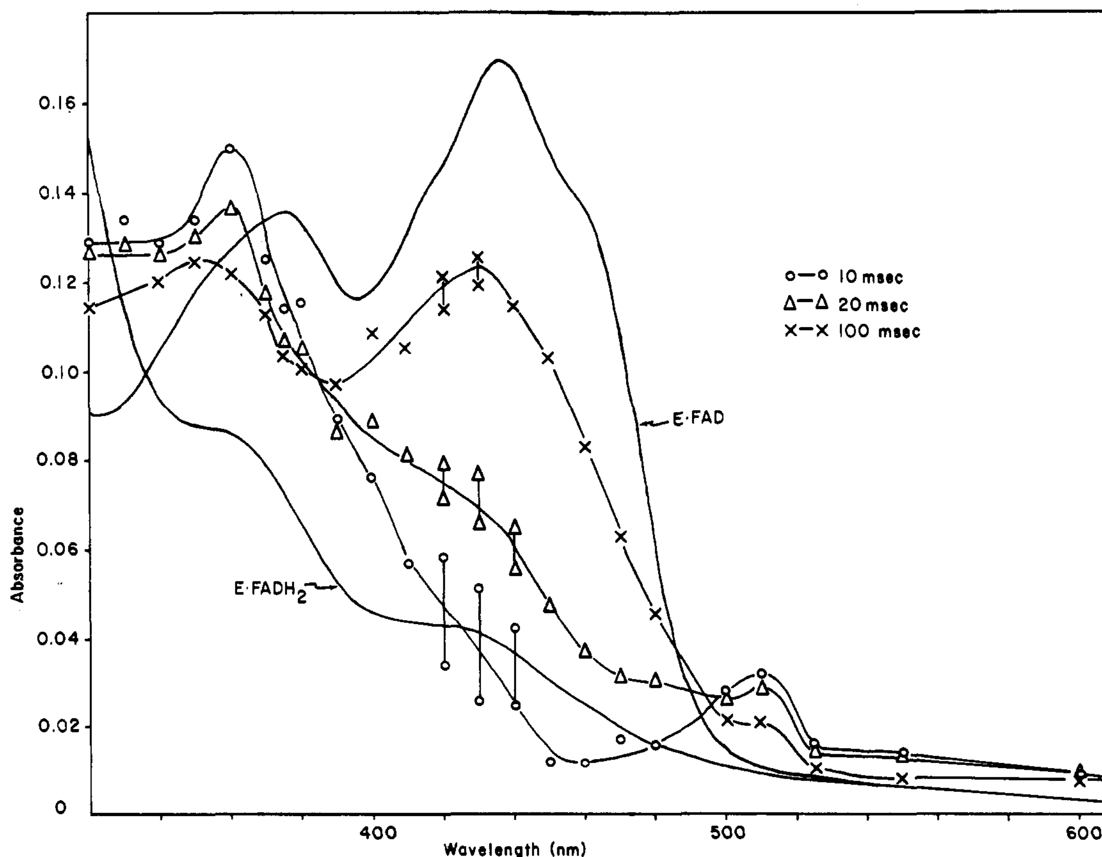


FIGURE 3: Time course spectra generated in an assay with reduced cyclohexanone oxygenase (0.014 mM) in complex with cyclohexanone (0.097 mM) reacted with oxygen (0.60 mM). E·$FADH_2$ indicates the spectrum of the assay solution before mixing with oxygen. E·FAD indicates the spectrum of the solution at the end of the reaction ($\sim$1 min after mixing). These assays were carried out at pH 7.2 and 4 °C as described under Methods.

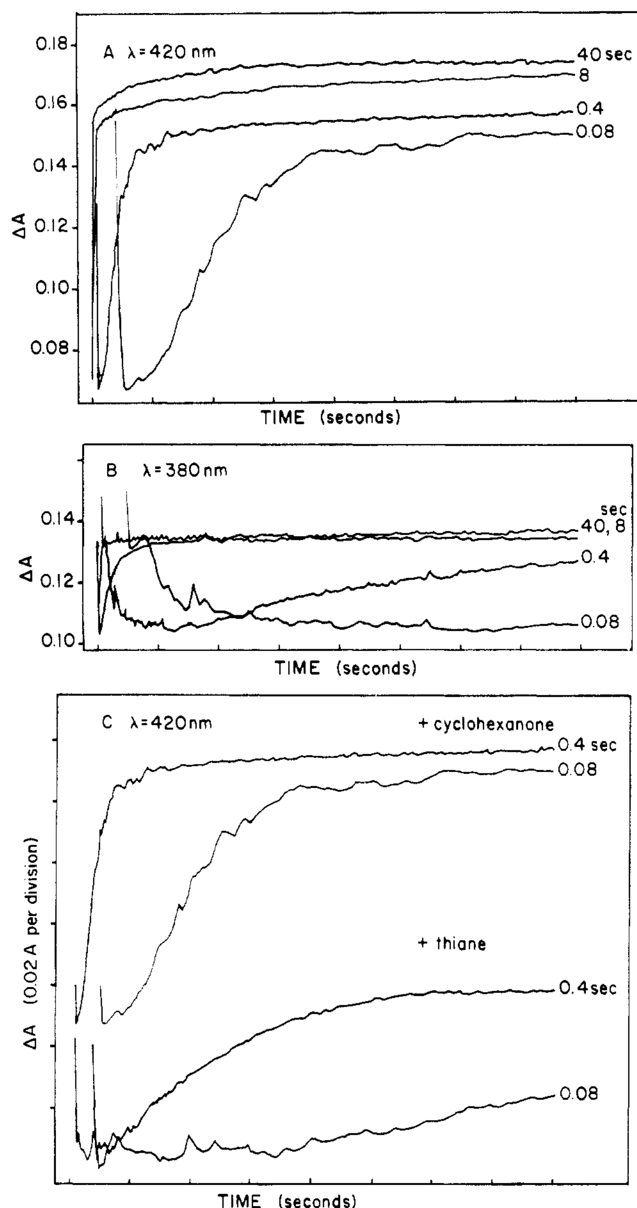
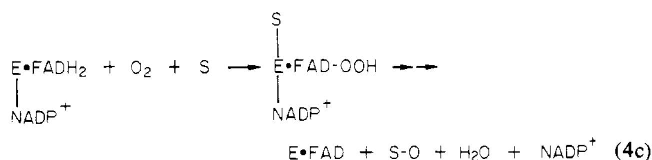


FIGURE 4: (A) These traces were recorded at pH 7.2 and 4 °C as described under Methods. Time course of absorption at 420 nm upon reacting oxygen (0.60 mM) with reduced cyclohexanone oxygenase (0.14 mM) in complex with cyclohexanone. Such assays as these are used to determine the spectra shown in Figure 3. (B) Same as (A) at 380 nm. (C) Time course of absorption at 420 nm upon reacting oxygen (0.6 mM) with reduced cyclohexanone oxygenase in complex with either cyclohexanone (0.097 mM) or thiane (0.29 mM). Note that the reaction in the presence of thiane is slower than that with cyclohexanone.

in the presence of $NADP^+$ (0.19 mM) at pH 7.2 and 4 °C (eq 4c). The spectrum of the proposed enzyme-bound 4a-



peroxyflavin intermediate, again formed within the mixing time ($k > 1000$ s^{-1}) during reaction with cyclohexanone, is shown in Figure 3. The same intermediate is formed in the presence of thiane. The fact that there is no obvious isosbestic point in the spectra indicates that more than two species are in solution at any one time. The nature of such species present in addition to the 4a-peroxyflavin intermediate and the final oxidized flavin is as yet undeciphered.

The reaction rate data are multiphasic and rather complex as is indicated by Figure 4A,B. The decay of the flavin–oxygen intermediate can be noted at λ 380 nm in Figure 4B. In the reoxidation of enzyme-bound flavin, monitored at 420 nm (Figure 4C), the first phase associated with an increase in absorbance is slower in the presence of thiane (k = 33.4 ± 6 s^{-1}) than with cyclohexanone (54.9–75.3 s^{-1}) and proceeds after a short, but detectable, lag (5–15 ms). The slowest step in the reaction is in a later phase (~1 s after mixing) and is k = 0.52 s^{-1} for the cyclohexanone oxygenation and k = 0.34 s^{-1} for the thiane oxidation, and may involve a charge-transfer complex or may even be product release. It is this step which chiefly limits overall turnover, as is noted by comparison with the steady-state V_{max} = 0.49 s^{-1} at pH 7.2 and 4 °C (Table II) with cyclohexanone. When [2,2,6,6-$^2H_4$]cyclohexanone was tested as a substrate, no deuterium kinetic isotope effect was observed in any phase of the oxygenase reaction.

We cannot separate out the formation and decay of individual intermediates, but it is obvious from examining Figure 3 that at least two intermediates are present during catalysis, and they are most likely the 4a-peroxyflavin and the 4a-hydroxyflavin (Entsch et al., 1976a). The nature of the species resulting in absorbance at 500–520 nm has not yet been determined.

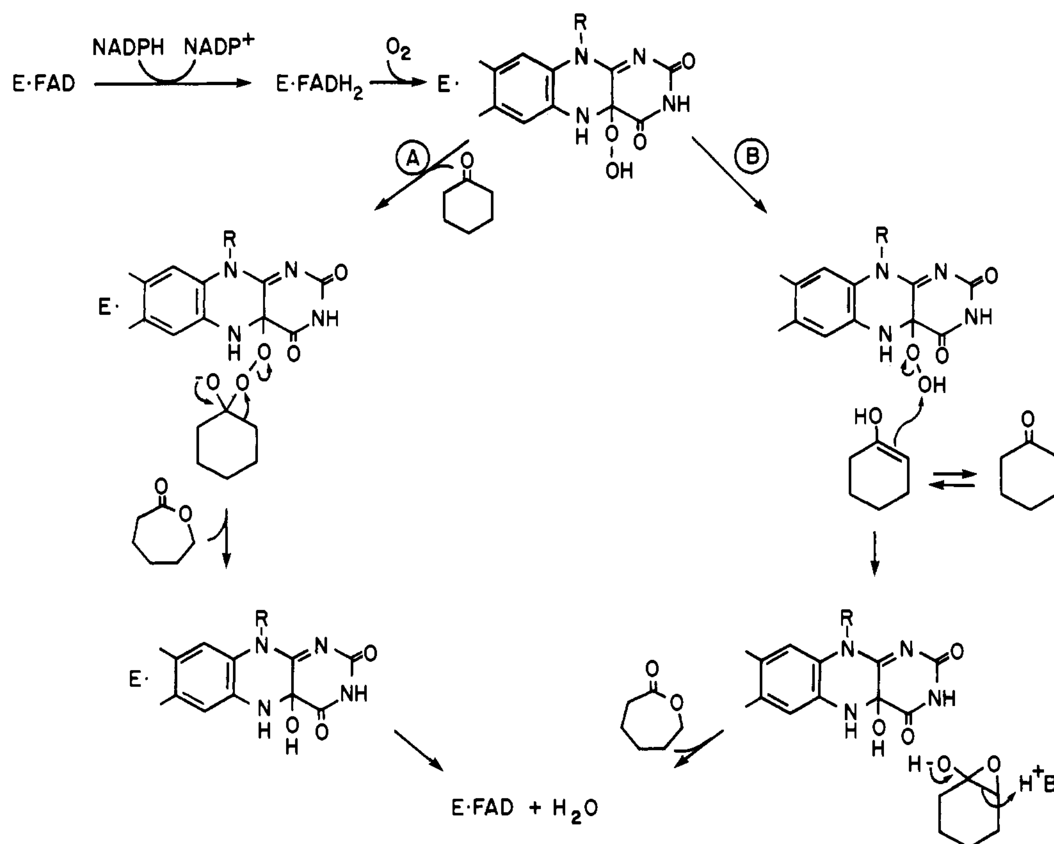
Free H_2O_2 Does Not Catalyze the Cyclohexanone Oxygenase Reaction. It seemed possible, if unlikely, that the 4a-peroxyflavin breaks down at the active site to form H_2O_2 and oxidized FAD and that the H_2O_2 thus formed is the actual oxygenating species in a classical Baeyer–Villiger sequence. The following experiment was performed in order to test this possibility. An anaerobic assay mixture containing cyclohexanone oxygenase (0.028 μM), $NADP^+$ (0.19 mM), H_2O_2 (0.18 mM), and [1-^{14}C]cyclohexanone (274 mM) at pH 9.0 was incubated at 30 °C as described under Methods. This incubation should allow for the generation of the E·FAD–H_2O_2–$NADP^+$ complex which might be needed for the oxygenation reaction to occur. At the end of 30 min, analysis revealed that only 0.50% of the expected (if NADPH and O_2 had been present) product lactone was produced, thus indicating that cyclohexanone oxygenase does not appear to catalyze oxygenative insertion on cyclohexanone with exogenous H_2O_2.[2]

It should be noted that in the bacterial monooxygenase luciferase, which carries out an apparent Baeyer–Villiger reaction converting RCHO to RCOOH, H_2O_2 when added to the oxidized enzyme does form the 4a-peroxide (Hastings et al., 1979). It is the peroxide, however, not free H_2O_2, which is involved in the oxygenation reaction. Cyclohexanone oxygenase simply may not have the kinetic ability to carry out the production of 4a-peroxide in a reverse direction from H_2O_2 and oxidized enzyme.

[2,2,6,6-$^2H_4$]Cyclohexanone Generates Tetradeuteriolactone. Chemical and enzymic precedents are available which indicate that the 4a-peroxyflavin is capable of oxygenating

[2] It should be noted, however, that the possibility still exists that the enzyme active site is not accessible to H_2O_2 free in solution and that during catalysis H_2O_2 is generated within the active site and is quickly captured by cyclohexanone. Although this point must be considered, it seems unlikely for the following reasons: (1) cyclohexanone is not an efficient scavenger of H_2O_2 in aqueous solution under conditions favorable for enzymatic catalysis; (2) the active site of cyclohexanone oxygenase seems to be fairly accessible—if one considers the broad substrate specificity, H_2O_2 should certainly be small enough to procure entry; and (3) H_2O_2 might leak out of the active site on occasion with slow substrates and be detected by catalase—this possibility has been tested with no measureable release of H_2O_2 in agreement with Donoghue et al. (1976).

Scheme III



sulfides and amines to their respective sulfoxides and *N*-oxides (Ball & Bruice, 1979, 1980). No precedents have yet been forwarded, however, which demonstrate that the 4a-peroxyflavin acts as a Baeyer–Villiger reagent to carry out oxygen insertion reactions on ketones.

In the reaction of cyclohexanone with the proposed 4a-peroxyflavin intermediate, it is possible to write mechanisms where the role of the ketone would be as an electrophile at the carbonyl carbon (Scheme IIIA) or as a nucleophile through the intermediacy of an enolate tautomer (Scheme IIIB). The following experimental evidence supports the reaction of the ketone as an electrophile. In an assay with cyclohexanone oxygenase and [2,2,6,6-$^2H_4$]cyclohexanone, unreacted ketone and the corresponding $^2H_4$-labeled lactone product were recovered by extraction with chloroform and subjected to GLC–MS analysis. Analysis of the product lactone showed it to have a relative intensity at m/e 118 equivalent to that of the starting ketone at m/e 102. There was less than 5% exchange of deuterium from either substrate or product. This result is in agreement with a recent report which involves proton NMR analysis of the lactone product from an incubation of [2,2,6,6-$^2H_4$]cyclohexanone with the enzyme (Schwab, 1981). In addition, [2,2,6,6-$^2H_4$]cyclohexanone shows no kinetic isotope effect in steady-state or in pre-steady-state assays; an enzymic enolization step would necessitate a C_2–H or C_6–H cleavage step. It is unlikely, in light of this evidence, that cyclohexanone reacts through an enolate (as in Scheme IIIB).[3]

[3] The enolate, however, could be generated in a fast step in catalysis where the proton removed from C-2 is sequestered within the enzyme active site and completely returned to that same position in the product after oxygen insertion–ring expansion. Sequestered protons are indicated at the active sites of D-amino acid oxidase (Bright & Porter, 1975; Porter et al., 1977; Walsh et al., 1973; Cheung & Walsh, 1976), *p*-hydroxybenzoate hydroxylase (Entsch et al., 1976a), and a bacterial flavoprotein transhydrogenase (Louie & Kaplan, 1970).

Table IV: Apocyclohexanone Oxygenase Reconstitution with Flavin Analogues[a]

FAD analogue	$E^{\circ\prime}$ [b] (mV)	K_{mapp} (nM)	V_{max} (units/mg)	rel V_{max}	% coupling[c]
7-chloro[d]	−128	189	8.0	0.44	ND
9-aza	−135	133	16.0	0.87	ND
FAD	−219	125	18.4	1.0	100
6-methyl[e]	−219	119		0.20	ND
1-deaza	−280	43500	18.4	1.0[f]	100
5-deaza	−311	62[g]	0	0	none

[a] Assays were conducted as described under Methods. All determinations were carried out with the same apoenzyme preparation unless otherwise specified. [b] The redox potentials shown here were reported by Spencer (1978). [c] Percent coupling indicates the extent to which NADPH oxidation is coupled with oxygen uptake during turnover. Some values were not determined (ND). [d] This analogue is 7-chloro-8-demethyl-FAD. [e] These values were determined in assays with apoenzyme which was 2 months old. The value for relative V_{max} then may be too low. [f] This is an average value for three separate experiments (with values ranging from 0.67 to 1.60) with three different preparations of apoenzyme. [g] This is the K_I for 5-deaza-FAD. This analogue is incompetent in O_2 reactivity and shows competitive inhibition against FAD in this system. This is an average of two determinations with apoenzyme preparations both different than that used in the other experiments; values ranged from 30 to 93 nM.

Apoenzyme Preparation and Reconstitution with Flavin Analogues. The apoprotein generated by KBr analysis of cyclohexanone oxygenase is a stable enzyme form and can be stored for up to 1 month at 4 °C and still be 100% reconstituted. Less than 10% of the enzyme molecules are bound to residual flavin based on reconstitutable activity; incubation of the apoprotein with FAD reconstitutes 100% of the holoenzyme activity. Assays for apoenzyme activity are routinely carried out at 30 °C in the presence of excess FAD (or FAD analogue). Attempts to reload the apoprotein with FAD or

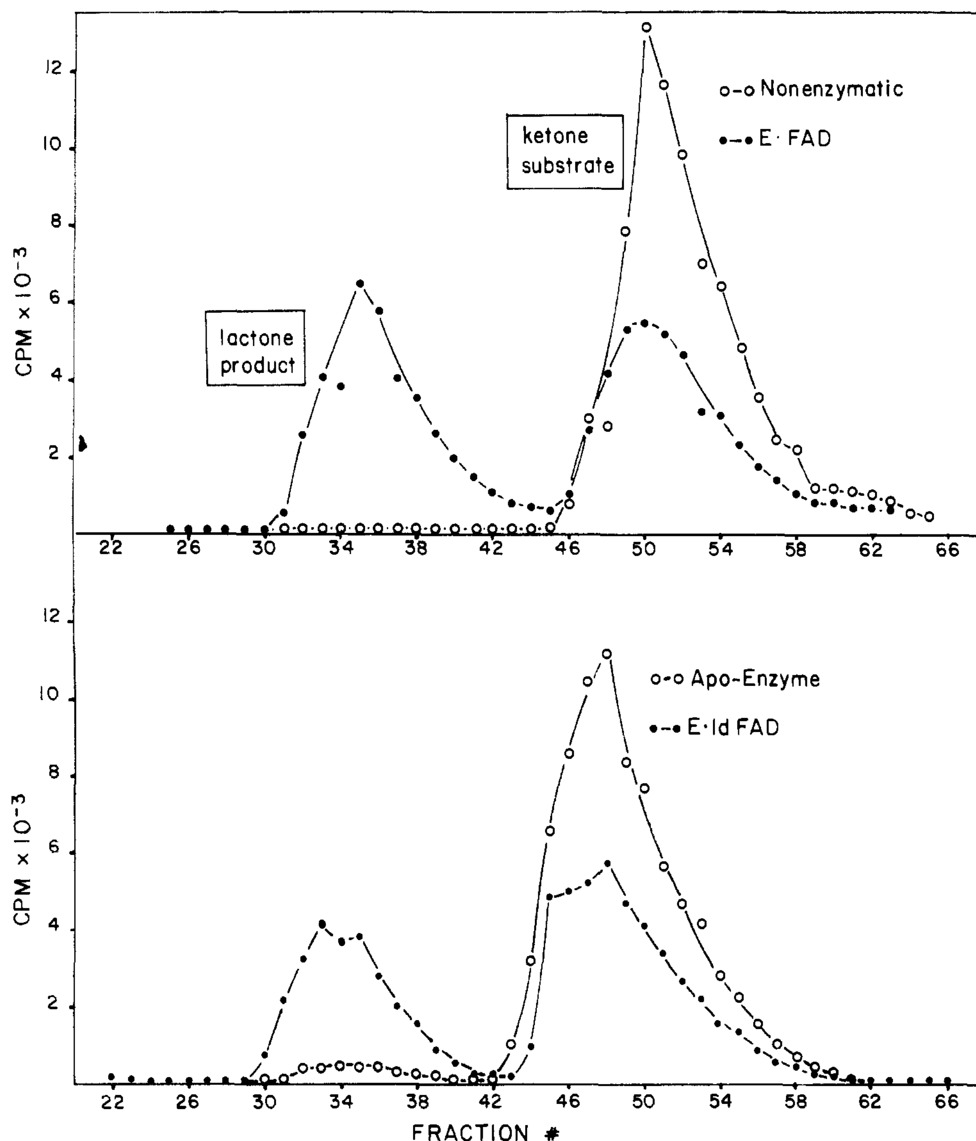


FIGURE 5: HPLC separation of cyclohexanone and ε-caprolactone. [1-^{14}C]Cyclohexanone (1 nmol of ^{14}C = 2000 cpm) was incubated in assays containing (top panel) (a) no enzyme or (b) cyclohexanone oxygenase and (bottom panel) (a) apocyclohexanone oxygenase or (b) 1-deaza-FAD-reconstituted apocyclohexanone oxygenase. The assay mixtures were treated as described under Methods and then injected onto a reverse-phase HPLC column. Any ^{14}C-labeled lactone produced is indicated by the appearance of radioactivity under the lactone peak. Fractions (300 μL) were collected and counted. See also Table V.

1-deaza-FAD followed by Sephadex G-25 column chromatography gave fairly poor yields of holoenzyme. This was attributed to the high binding constants for FAD and FAD analogues with this apoprotein (see Table IV).

In addition to FAD, several FAD analogues were assayed for activity with apocyclohexanone oxygenase. Apparent K_m and V_{max} values were determined in steady-state assays and are shown in Table IV. In addition to those analogues which are catalytically active, 5-deaza-FAD [catalytically inactive in oxygen reactions (Spencer et al., 1979)] was assayed for inhibition of FAD-reconstituted activity. A K_I = 62 nM for 5-deaza-FAD as a competitive inhibitor was thereby established.

The last column in Table IV shows data for the percent coupling of NADPH oxidation with oxygen transfer during steady-state turnover. In particular, both FAD and 1-deaza-FAD, when reconstituted with apocyclohexanone oxygenase, are fully coupled in turnover (Table V, Figure 5). In the absence of enzyme, no ^{14}C-labeled lactone was produced. The catalytic competence of 1-dFAD–cyclohexanone oxygenase to oxygenate substrate is in marked contrast to 1-dFAD-reconstituted phenolic monooxygenases which yield uncoupled NADPH oxidation with all O_2 flux converted to H_2O_2 (Entsch et al., 1980).

Table V: Stoichiometry of Cyclohexanone Oxygenase Reaction[a]

enzyme complex	NADPH oxidized (nmol)	lactone produced (nmol)
E·FAD	17	15
	15	19
E·1-dFAD	15	15
	10	10
apoenzyme	2	1.4

[a] These values were determined from data such as shown in Figure 5. NADPH consumption was monitored spectrophotometrically (A_{340}). The amount of lactone produced was quantitated by the level of ^{14}C-labeled product which appears following reaction of [1-^{14}C]cyclohexanone with cyclohexanone oxygenase as shown in Figure 5. See Methods for assay conditions.

Discussion

The results presented in this paper document that cyclohexanone oxygenase behaves as a typical bacterial flavoprotein monooxygenase in the sense that it forms a detectable FAD–

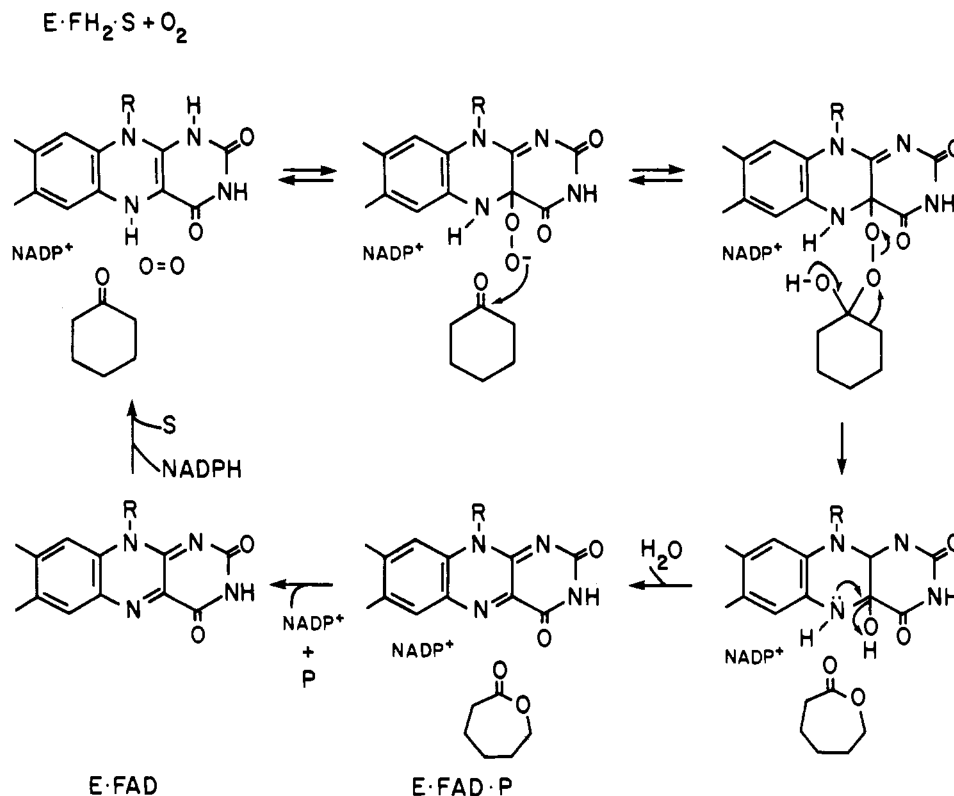
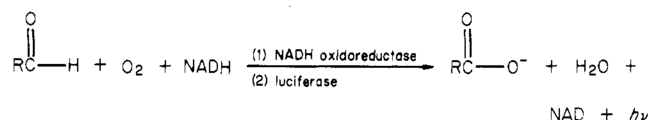


FIGURE 6: Proposed mechanism for the cyclohexanone oxygenase catalyzed oxygen insertion reaction with cyclohexanone (S) to yield as product ε-caprolactone (P). This mechanism involves both the 4a-peroxyflavin and the 4a-hydroxyflavin intermediates.

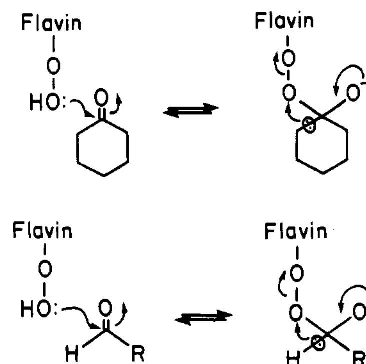
4a-hydroperoxide as a transient intermediate. This flavin hydroperoxide is now proposed to be the oxygen transfer agent, or progenitor thereof, for insertion of the distal oxygen atom into a carbon–carbon bond of cyclic ketones to form the corresponding ring-expanded lactones (Figure 6) (Walsh et al., 1980). Similarly, oxygen transfer to a sulfide produces a sulfoxide. This is distinct from aromatic ring hydroxylases where oxygen is inserted, in a formal sense, into a C–H bond (Walsh, 1979).

The precedent in model chemistry is the Baeyer–Villiger reaction which involves a nucleophilic attack by an alkyl hydroperoxide on a ketone. The tetradeuteriocyclohexanone to tetradeuterio-ε-caprolactone result strongly supports this mechanistic paradigm (Scheme III, route A), rather than the enolate-epoxidation alternative (route B) in which the nucleophile is the ketone substrate. This general reaction type is well represented among biological reactions, e.g., 2,5-bornanedione → lactone, 2-tridecanone → undecyl acetate, acetophenone → phenyl acetate (Dagley, 1978), some of which may be catalyzed by flavoprotein monooxygenases. A multimeric ketolactonizing system has been reported by Trudgill et al. (1966), and in this case, an iron-containing protein carries out the oxygen transfer reaction.

The catalytic mechanism of cyclohexanone oxygenase can be compared with three other types of flavoprotein monooxygenases which also form 4a-hydroperoxyflavin intermediates. The first is bacterial luciferase (Hastings et al., 1973a,b; Hastings & Balny, 1975), and at first glance, this protein seems mechanistically related, particularly when coupling with the enzyme NADH oxidoreductase from the same marine bacteria (Fisher & Walsh, 1974). The oxidation of a long-chain aldehyde to an acid can be construed as a



Baeyer–Villiger reaction on an aldehyde (Eberhard & Hastings, 1972), rather than on a cyclic ketone. In each case, the distal oxygen transferred from a bound flavin hydroperoxide would react as a nucleophilic oxygen atom attacking an electrophilic carbonyl carbon in the cosubstrate. In the ketone

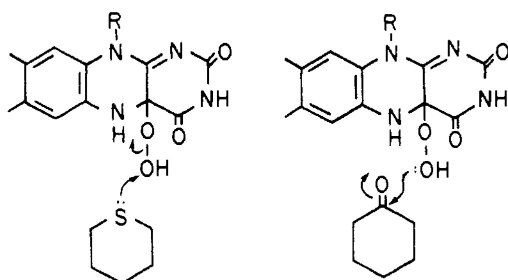


case, the tetrahedral adduct decomposes toward product with C–C bond migration to expel the 4a-hydroxyflavin. A corresponding route in luciferase would be migration of the C_1-H of the aldehyde substrate as a hydride ion. A major distinction, at least in product outcome from luciferase and cyclohexanone oxygenase action, is that only the former yields a photon (10% quantum yield) (Becvar et al., 1976; Hastings & Nealson, 1977). Our analyses fail to detect any light from the cyclohexanone oxygenase catalyzed reaction. The molecular bases for these distinctions are unknown.

Comparison of cyclohexanone oxygenase with the well-studied aromatic ring hydroxylases reveals some similarities as well, despite the distinction that the phenol → catechol conversions are best interpreted as delivery of an electrophilic oxygen equivalent (from a flavin hydroperoxide) to a nucleophilic substrate site, just the reverse of the polarity described above. Moreover, Entsch et al. (1976b) reported that *p*-hydroxybenzoate hydroxylase processed *p*-mercaptobenzoate

to the disulfide in an O_2-linked process; this was interpreted as involving enzymic oxygenation to form a sulfenate product which rapidly reacted with a second substrate molecule to yield the observed disulfide and H_2O. The S-oxygenation sequence most probably involves the substrate sulfur atom as the nucleophile and flavin hydroperoxide as the electrophile.

The cyclohexanone oxygenase can also carry out S-oxygenation, and, on a cyclic sulfide substrate (thiane), the product is not an unstable sulfenate but a stable and isolatable sulfoxide (thiane 1-oxide). Cyclohexanone oxygenase is so far unique among flavoprotein monooxygenases in demonstrated ability to deliver an oxygen atom derived from O_2 to both a nucleophilic and an electrophilic substrate. Thus, it provides a clear demonstration that the enzymatically generated 4a-hydroperoxyflavin, assuming that it is the actual oxygen transfer agent, is an ambident reagent, capable of delivering either electrophilic or nucleophilic oxygen. The nature of the peroxide O–O bond cleavage is controlled by the electron availability in the cosubstrate to be oxygenated.



The third variant of flavoprotein monooxygenase to be compared is the only mammalian representative, the liver microsomal FAD-containing monooxygenase from hog liver, which carries out oxygenation at nitrogen or sulfur (Ziegler, 1980). This enzyme has broad specificity for amines and sulfur-containing substrates and is unusual in that the FAD 4a-OOH accumulates in steady state (in the absence of substrate). This species binds the substrate and effects a rapid oxygenation, followed by product release which occurs at nearly the same rate (which is $\sim V_{max}$) for all substrates (Poulsen & Ziegler, 1979; Beaty & Ballou, 1981a,b). The reduction of this enzyme-bound FAD by NADPH is not accelerated by cosubstrates, similar to the cyclohexanone oxygenase pre-steady-state results obtained here (Beaty & Ballou, 1981a) and in sharp distinction to the aromatic hydroxylases where 10^4-fold accelerations in FAD reduction are effected by the presence of cosubstrate (Massey & Hemmerich, 1978). It is the mechanistic elucidation of this *S,N*-oxidase and of the sulfur oxidations of cyclohexanone oxygenase that relates the enzymatic reactions directly with the nonenzymatic model chemistry of the N_5-alkylated flavin peroxides. To date, these N_5-alkylated flavin peroxides have been shown to be competent only in S- and N-oxidation, but not in ketone lactonization or aromatic ring hydroxylation (Ball & Bruice, 1979, 1980).

A final point of comparison between cyclohexanone oxygenase and *p*-hydroxybenzoate hydroxylase is the catalytic competence of 1-deaza-FAD-reconstituted enzymes to carry out oxygen transfers. It has previously been noted that 1-deaza-FAD–*p*-hydroxybenzoate hydroxylase has a V_{max} for NADPH consumption that is 25% that of FAD–enzyme but that all O_2 flux is to H_2O_2 (Entsch et al., 1980). No oxygenation of substrate occurs, so that only an oxidase activity ensues. However, with 1-deaza-FAD–cyclohexanone oxygenase, there is 100% coupling of NADPH oxidation to ketone oxygenation [see also Walsh et al. (1980)]. Thus, 1-deaza-FAD is fully competent to act as an oxygen transfer agent. The inability of the *p*-hydroxybenzoate hydroxylase–1-deaza-FAD complex to oxygenate substrates may be a kinetic problem of intramolecular breakdown to 1-deaza-FAD and H_2O_2 before oxygen transfer can compete. In this regard, model studies by Bruice and colleagues show that 5-ethyl-1-deazalumiflavin is competent for oxygen transfer to amines and sulfur atoms, but at rates 3–10 times slower than with the normal lumiflavin and analogue itself.[4]

The 1-deaza-FAD system is, then, a fully competent flavin coenzyme analogue but one with a redox potential some 70 mV more negative than that for FAD (−210 mV), allowing one to alter the internal thermodynamics of the reconstituted flavoenzymes to probe mechanism (Spence et al., 1976). It remains to be seen where the rate-limiting steps in this biological Baeyer–Villiger oxygenation process are and what the specificity of cyclohexanone oxygenase is with both ketone and sulfide substrates. Further work is required to enumerate the spectral intermediates and to elucidate their structural characteristics (Figure 3) and their kinetic role in the catalytic sequence.

Acknowledgments

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