triethylene glycol. To this solution were added 23 mg of K_2CO_3 and 25 μL of hydrazine hydrate. This mixture was heated at 180 °C (bath temperature) under N_2 for 1 h, at which point another 25 μL of hydrazine hydrate was added and the temperature was raised to 200 °C. After 30 min, the solution was heated to 250 °C for 2 h. The mixture was cooled and diluted with water. This mixture was extracted with ether, and the ether solution was washed with 1 N HCl followed by brine and dried (Mg_4SO_4). Concentration gave 10 mg of crude product consisting of a 93:7 mixture of two compounds, as determined by capillary GLC analysis. The minor product was tentatively identified by GC–MS analysis as dihydrosilphinene (M^+ 206). The major product was silphinene (M^+ 204). The identity of the major product was confirmed by comparison of a high-field ^1H NMR spectrum with the spectrum of authentic silphinene.[17] In addition, the ^{13}C NMR matched the reported spectrum.[5] MS, *m/e* calcd for $C_{15}H_{24}$, 204.1878; found 204.1876. The yield of (±)-silphinene was 93%.

Acknowledgment. We acknowledge financial support from the donors of the Petroleum Research Fund, administered by the American Chemical Society. We also thank Professor Leo Paquette for sending an ^1H NMR spectrum of silphinene for comparison.

Registry No. (±)-**11**, 83057-81-2; **14**, 87937-90-4; (±)-**15a**, 87648-96-2; (±)-**15b**, 95121-99-6; (±)-(2α)-**16a**, 87649-04-5; (±)-(2β)-**16a**, 87680-59-9; (±)-(2β)-**16b**, 95122-00-2; (±)-**17**, 95190-94-6; (±)-**18**, 87649-02-3; (±)-(2α)-**19**, 95122-01-3; (±)-(2β)-**19**, 95122-02-4; (±)-**20a**, 95122-03-5; (±)-**20b**, 95122-04-6; (±)-**21**, 95122-05-7; (±)-**22**, 95122-06-8; (±)-**23**, 95122-07-9; **24**, 95122-08-0; (±)-**25**, 95122-09-1; $EtOCH{=}CH_2$, 109-92-2; $Li(CH_3)C{=}CH_2$, 6386-71-6.

Functional Group Diversity in Enzymatic Oxygenation Reactions Catalyzed by Bacterial Flavin-Containing Cyclohexanone Oxygenase

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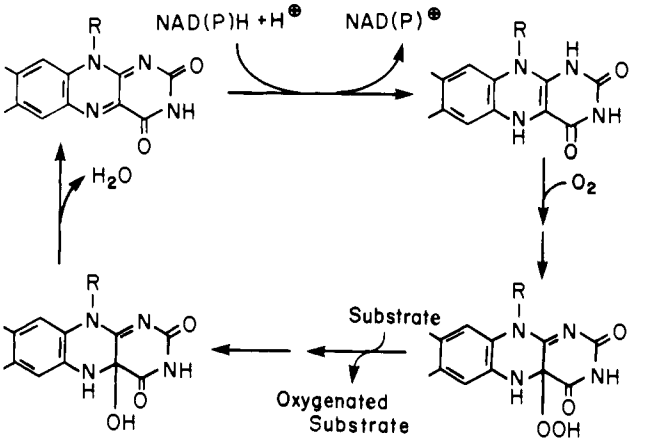
Abstract: The bacterial flavoprotein monooxygenase cyclohexanone oxygenase was found by spectrophotometric NADPH consumption assays and product analysis studies to perform oxygenation reactions on ketones, aldehydes, sulfides, selenides, boronic acids, a phosphite ester, and an iodide ion. Kinetic parameters (K_m, V_{max}) are reported for these substrates. The relevance of these results to possible active oxygen-transfer species in this enzyme is discussed. The potential utility of boronic acids as general probes for nucleophilic oxygen-transfer capability in oxygenases and in model chemistry is analyzed. The potential utility of cyclohexanone oxygenase as an enatioselective and/or chemoselective oxidant for organic molecules is assessed. Unsuccessful attempts at exploiting the 2,3-sigmatropic rearrangement of allyl sulfoxides and allyl selenoxides for mechanism-based inactivation of cyclohexanone oxygenase are reported. The use of the facile 2,3-sigmatropic rearrangement of allyl selenoxides to generate electrophilic allyl selenates for the design of mechanism-based inactivators for other enzymes is proposed.

The set of enzymes classified as monooxygenases,[2] transferring one atom from O_2 to specific cosubstrates, utilize various cofactors, including flavins, pterins, copper, and iron-containing heme (cytochromes P-450). In the past decade much progress has been made in the analysis of the nature of the enzyme-bound active oxygen-transfer species in these enzymes.[3]

A variety of flavoprotein monooxygenases are known which oxygenate various substrate molecules by catalyzing the four-electron reduction of dioxygen with two electrons derived from reduced nicotinamide cofactor and two electrons derived from substrate. Flavoprotein monooxygenases can be classified according to the "natural" substrates oxygenated and include phenolic aromatic α-hydroxylases (conversion of phenols to catechols),[4] mammalian microsomal sulfur–nitrogen oxygenases,[5] bacterial luciferases (conversion of straight-chain aliphatic aldehydes to straight-chain aliphatic carboxylic acids + light),[6] and cyclic ketone oxygenases such as cyclohexanone oxygenase (conversion of cyclic ketones to lactones) (eq 1).[7]

Scheme I



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(2) Walsh, C. "Enzymatic Reaction Mechanisms"; W. H. Freeman: San Francisco, 1979; pp 411–431, 458–485.

(3) Recent reviews include: (a) White, R. E., Coon, M. J. *Annu. Rev. Biochem.* **1980**, *49*, 315–356. (b) Malmstrom, B. G. *Annu. Rev. Biochem.* **1982**, *51*, 21–59. (c) Ballou, D. P. In "Flavins and Flavoproteins"; Massey, V., Williams, C. H., Jr., Eds.; Elsevier: New York, 1982; pp 301–310.

(4) (a) Detmer, K.; Massey, V.; Ballou, D. P.; Neujahr, H. Y. In "Flavins and Flavoproteins"; Massey, V., Williams, C. H., Jr., Eds.; Elsevier: New York, 1982; pp 335–358 and references therein. (b) References in ref 8. (c) Entsch, B.; Ballou, D. P.; Massey, V. *J. Biol. Chem.* **1976**, *251*, 2550–2563. (d) Husain, M.; Massey, V. *J. Biol. Chem.* **1979**, *254*, 6657–6666.

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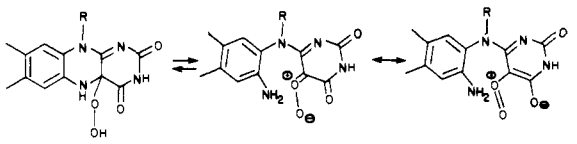
A minimum catalytic cycle for the flavin cofactor in all these flavoprotein monooxygenases is shown in Scheme I. The proposed structures for the various intermediates have been assigned by comparison of the UV–visible spectral data of intermediates observed in single turnover stopped-flow rapid kinetic studies with data on stable model compounds.[3,8] Evidence from stopped-flow

$$\text{Substrate} + \text{NAD(P)H} + \text{H}^{\oplus} + \text{O}_2 \xrightarrow[\text{Monooxygenase}]{\text{Flavoprotein}} \text{Oxygenated Substrate} + \text{NAD(P)}^{\oplus} + \text{H}_2\text{O} \quad (1)$$

spectral studies has been presented for the intermediacy of a flavin 4a-hydroperoxide (4a-Fl-OOH) in the catalytic cycle of numerous flavoprotein monooxygenases including melilotate hydroxylase, salicylate hydroxylase, *p*-hydroxybenzoate hydroxylase, phenol hydroxylase, cyclohexanone oxygenase, and mammalian sulfur–nitrogen oxygenase.[4,5] In the case of bacterial luciferase a stable enzyme-bound 4a-Fl-OOH has been isolated by low-temperature chromatography and characterized by UV–visible and ^{13}C NMR spectroscopy.[6b]

It was proposed several years ago that the 4a-Fl-OOH intermediate could not be the active oxygen-transfer species in flavoprotein monooxygenases since alkyl hydroperoxides are not hydroxylating agents in the absence of metal ion catalysts and flavoprotein monooxygenases do not contain metal ions.[9] The carbonyl oxide tautomer of the 4a-Fl-OOH (essentially a vinylogous ozone) was invoked as a transient, highly reactive oxygen-transfer reagent for oxene (oxygen atom) transfer to the substrate (eq 2). Other structures have been proposed for the



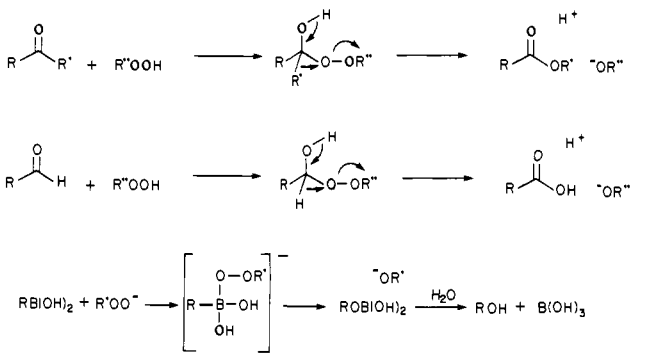
(2)

transient-active oxygen-transfer species derived from the known 4a-Fl-OOH.[9c] A recent model study does not support the carbonyl oxide mechanism.[8b]

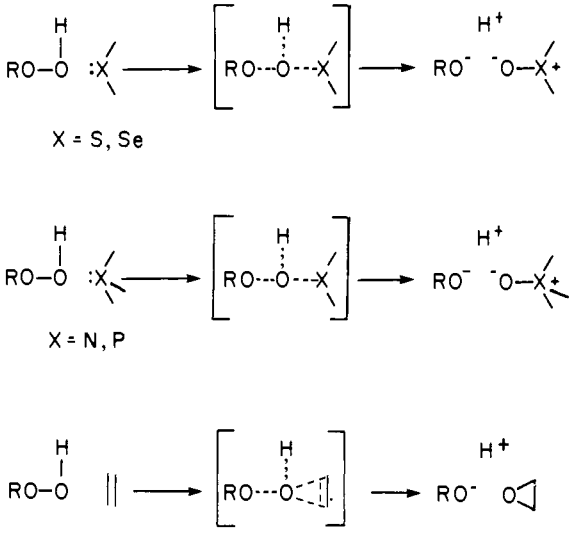
The proposal that a 4a-Fl-OOH could not be an effective hydroxylating agent was based on the assumption that a 4a-Fl-OOH possesses the chemical properties of a typical alkyl hydroperoxide.

With the established analogy between 4a-Fl-OOH model compounds and peracids for sulfur, nitrogen, and iodide oxidation, the fact that peracids are known to hydroxylate phenols[10a–c] and even unactivated aromatic rings[10d] suggests that peracids may represent reasonable chemical models for flavin monooxygenase-mediated phenolic aromatic hydroxylations. The detailed mechanisms of hydroxylations require close scrutiny, however, as illustrated in recent evidence that peracid epoxidations

Scheme II



Scheme III



of olefins and oxygenations of aromatic rings occur via free radical or two-electron mechanisms under different conditions.[10f] The only flavin hydroperoxide model reaction which has been shown to involve phenol hydroxylation is the reaction of the anion of a flavin 4a-hydroperoxide with a phenolate anion. This has been shown to be a dioxygen-transfer reaction followed by elimination of H_2O and reduction of the quinone intermediate by dihydroflavin intermediate. It has been proposed that the reaction may proceed through a flavin 4a,10a-dioxetane as a means to activate the flavin 4a-hydroperoxide for oxygen transfer.[10g]

In opposition to the idea that model chemistry in solution may be relevant to flavoprotein monooxygenase chemistry, Visser has recently proposed that unique features of the active site of *p*-hydroxybenzoate hydroxylase allow the enzyme to do chemistry which cannot occur in solution.[11] From an examination of X-ray crystallographic data on *p*-hydroxybenzoate hydroxylase, Visser proposes that an enzyme-bound, enzyme-stabilized flavin 4,4a-dioxetane derivative is the active oxygen-transfer species in a reaction which has no known counterpart in model organic chemistry solution. No attempt was made in the proposals to account for intermediate II observed by Massey and colleagues.[4c]

The concept of catalysis of oxygen transfer via a transient highly reactive species derived from the 4a-Fl-OOH was introduced from a consideration of phenolic aromatic hydroxylases.[4c,9a,b] Whether such catalysis occurs in the other types of flavoprotein monooxygenases remains unresolved and largely unaddressed.

Cyclohexanone oxygenase is an inducible bacterial flavoprotein monooxygenases produced by growth of *Acinetobacter* NCIB 9871[7] in the presence of cyclohexanol as the inducer. *Acinetobacter* NCIB 9871 was obtained by Trudgill by elective culture of soil samples screened for the ability to grow on cyclohexanol as the sole carbon source. The enzyme was first characterized

(7) Donoghue, N. A.; Norris, D. B.; Trudgill, P. W. *Eur. J. Biochem.* **1976**, *63*, 175–192. (b) Ryerson, C. C.; Ballou, D. P.; Walsh, C. *Biochemistry* **1982**, *21*, 2644–2655. (c) Light, D. R.; Waxman, D. J.; Walsh, C. *Biochemistry* **1982**, *21*, 2490–2498. (d) Schwab, J. M.; Li, W.-B.; Thomas, L. P. *J. Am. Chem. Soc.* **1983**, *105*, 4800–4808.

(8) (a) Bruice, T. C.; Noar, J. B.; Ball, S. S.; Venkataram, U. V. *J. Am. Chem. Soc.* **1983**, *105*, 2452–2463. See also: (b) Wessiak, A.; Bruice, T. C. *J. Am. Chem. Soc.* **1983**, *105*, 4809–4825.

(9) (a) Hamilton, G. A. In "Progress in Bioorganic Chemistry"; Kaiser, E. T., Kezdy, F. J., Eds.; Wiley-Interscience: New York, 1971; Vol. 1, pp 83–157, particularly 134–142. (b) Hamilton, G. A. In "Molecular Mechanisms of Oxygen Activation"; Hayashi, O., Ed.; Academic Press: New York, 1974; pp 405–451, particularly 431–435. (c) Contains a collection of and a discussion of several different proposed oxenoid intermediates derived from the 4a-Fl-OOH intermediate: Bruice, T. C. In "Biomimetic Chemistry"; Dolphin, D., McKenna, C., Murakami, Y., Tabushi, I., Eds.; American Chemical Society: Washington, DC, 1980; pp 88–118.

(10) (a) Whiting, D. A. In "Comprehensive Organic Chemistry"; Barton, D., Ollis, W. D., Stoddard, J. F., Eds.; Pergamon Press: Fairview Park, Elmsford, NY, 1979; Vol. 1, p 742. (b) Chambers, R. D.; Goggin, P.; Musgrave, W. K. A. *J. Chem. Soc.* **1959**, 1804–1807. (c) Bryce-Smith, D.; Gilbert, A. *J. Chem. Soc.*, **1964**, 873–876. (d) Hart, H. *Acc. Chem. Res.* **1971**, *4*, 337–343. (e) Sonovsky, R. In "Organic Peroxides"; Swern, D., Ed.; Wiley-Interscience: New York, 1970; Vol. 2, pp 319–323. (f) Fonouni, H. E.; Krishnan, S.; Kuhn, D. G.; Hamilton, G. A. *J. Am. Chem. Soc.* **1983**, *105*, 7672–7676 and references therein. (g) Muto, S.; Bruice, T. C. *J. Am. Chem. Soc.* **1982**, *104*, 2284–2290.

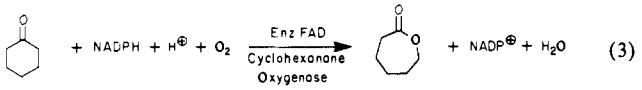
(11) Visser, C. M. *Eur. J. Biochem.* **1983**, *135*, 543–548.

Table I. Oxygenation Activities of Cyclohexanone Oxygenase in Which Formally Nucleophilic Oxygen Is Delivered to the Substrate

substrate	K_m,[a] μm	V_{max},[a,b] %	product[a,c]
cyclohexanone	6[d]	100[d]	6-hexanonlactone[d] (ε-caprolactone)
1-phenyl-2-propanone (phenylacetone)	330	7	benzyl acetate[e]
2-phenyl-1-ethanal	350	15	phenyl acetic acid (65%)[e,f,g] benzyl formate (12%)[e,f,g] benzyl alcohol (23%)[e,f,g]
butanal(butyraldehyde)	25	35	butanoic acid[e] (butyric acid)
phenylboronic acid	43	10	phenol[e,h]
n-butylboronic acid (1-butane boronic acid)	9	157	
n-octylboronic acid (1-octanylboronic acid)	2.5	145	1-octyl alcohol[e,h]

[a] See Experimental Section for details. [b] 100% = ca. 400 min^{-1}. [c] Reference 13. [d] Reference 7a. [e] Identified by ^1H NMR comparison with authentic material. [f] No control was run. [g] Relative ratios of products. [h] Identified by silica gel thin-layer chromatographic comparison with authentic material.

as oxidizing cyclohexanone to caprolactone in an apparent Baeyer–Villiger reaction (eq 3).[7a] Recent mechanistic studies



(3)

by Schwab[7d] on this cyclohexanone oxygenase have shown that the enzyme-catalyzed reaction exhibits several characteristic Baeyer–Villiger features such as retention of configuration at the migrating center in optically active 2-deuteriocyclohexanone and preferential migration of the more substituted center in 2-methylcyclohexanone.

The Baeyer–Villiger reaction is a typical reaction performed by peroxide-containing reagents.[12] Most oxygenation reactions performed by various flavoprotein monooxygenases[3-7] are typical reactions performed by peroxide-containing reagents. It is reasonable to assume, in the absence of any evidence to the contrary, that the 4a-Fl-OOH is chemically competent to perform these reactions (see Schemes II and III). The validity of such suppositions is significantly substantiated by Bruice's recent studies.[8a]

We have now surveyed cyclohexanone oxygenase for the ability to perform characteristic reactions performed by peroxide-containing reagents to assess how strongly the enzymatic oxygenation reactions parallel model reactions performed by peroxides and peracids. Reactions examined included those which has been observed in other flavoprotein monooxygenases as well as those which previously have had no known enzymatic counterpart.

Results and Discussion

Substrate Kinetic and Product Isolation Studies. Our results, summarized in Tables I and II along with two earlier results from our laboratory and one from Trudgill's, show that cyclohexanone oxygenase performs a wide range of typical reactions performed by peroxide-containing reagents.[12]

Initial rates of reaction vs. substrate consumption assays at 340 nm show that the enzyme exhibits simple saturation kinetics with all these substrates.

Since NADPH consumption assays do not unambiguously demonstrate that a compound is an oxygenatable substrate (e.g., it could be inducing an NADPH oxidase activity with O_2 converted to H_2O_2), product analysis studies were undertaken to determine unequivocally whether the molecules under study were true substrates for oxygen transfer. Since much enzyme was available (0.7 mg of homogeneous enzyme/1.0 g of cells) and some of the expected products did not possess useful chromophores for sensitive HPLC analysis with spectrophotometric detection, relatively large-scale incubations were performed to allow product isolation followed by identification by ^1H NMR and thin-layer chromatographic comparison with authentic products.

Table II. Oxygenation Activities of Cyclohexanone Oxygenase in Which Formally Electrophilic Oxygen Is Delivered to the Substrate

substrate	K_m,[a] μm	V_{max},[a,b] %	product[a,c]
thiane (pentamethylene sulfide, (tetrahydrothiopyran)	24[d]	87[d]	thiane sulfoxide[d]
ethyl *p*-tolyl sulfide	220[e]	26[e]	ethyl *p*-tolyl sulfoxide[e]
phenyl allyl sulfide	110	13	phenyl allyl sulfoxide[f,g]
thiane sulfoxide	178	60	thiane sulfone[f,g]
phenyl methyl selenide	44	130	phenyl methyl selenoxide[f,g]
phenyl allyl selenide	30	82	
triethyl phosphite	360	50	
iodide (sodium iodide)	2500	43	

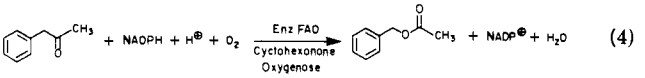
[a] See Experimental Section for details. [b] 100% = ca. 400 min^{-1}. [c] Reference 13. [d] Reference 7b. [e] Reference 7c. [f] Identified by ^1H NMR comparison with authentic material. [g] Identified by silica gel thin-layer chromatographic comparison with authentic material.

Cyclohexanone Oxygenase Catalyzes Different Types of Typical Oxygenation Reactions Performed by Peroxide-Containing Reagents. The oxygenation reactions performed by peroxide-containing reagents[12] (hydrogen peroxide, alkyl hydroperoxides, peracids, and cyclohexanane oxygenase) deliver either a formally nucleophilic or formally electrophilic oxygen atom to the substrate,[12f] as illustrated in Schemes II and III, respectively, using an alkyl hydroperoxide as the oxygen-transfer species.

The Baeyer–Villiger aldehyde and boronic acid oxygenations require initial attack of a nucleophilic oxygen on the carbonyl carbon or boron centers followed by rearrangement. In contrast the sulfide, selenide, phosphite ester, and iodide[14] oxygenations require the transfer of an electrophilic oxygen atom to a nucleophilic electron pair on the substrate. To our knowledge cyclohexanone oxygenase is the first example of an oxygenase enzyme capable of both nucleophilic or electrophilic oxygenation activities, as necessary for substrate oxygenation (Tables I and II).

Delivery of Nucleophilic Oxygen: Further Evidence for the Correspondence between the Enzymatic and Chemical Baeyer–Villiger Reaction. One of our results augments Schwab's results[7d] that the enzymatic Baeyer–Villiger reactions performed by cyclohexanone oxygenase exhibit characteristics of a Baeyer–Villiger reaction performed by peracids.

Our enzymic results with the acyclic ketone phenylacetone (Table I, eq 4), in which exclusive benzyl migration to form benzyl acetate occurs, are in accord with the migratory aptitude observed



(4)

in the Baeyer–Villiger reaction of phenylacetone performed by trifluoroperacetic acid.[15]

Delivery of Nucleophilic Oxygen: Oxygenation of Aldehyde Substrates. Aldehyde oxygenations by peracids are similar to

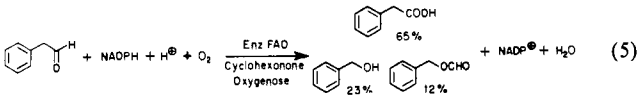
(12) Lee, J. B., Uff, B. C. *Quart. Rev.* **1967**, *21*, 429–457. (b) Rugero, C.; Edwards, J. C. "Organic Peroxides"; Swern, D., Ed.; Wiley-Interscience: New York, 1970; Vol. 1, pp 199–264. (c) Hiatt, R. "Organic Peroxides"; Swern, D., Ed.; Wiley-Interscience: New York, 1971; Vol. 2, pp 60–85. (d) Plesnicar, B. "Oxidation in Organic Chemistry"; Trahanovsky, W., Ed.; Academic Press: New York, 1978, Part C, pp 211–296. (e) Mimoun, H. *Angew. Chem., Int. Ed. Engl.* **1982**, *21*, 734–750. (f) Furia, F. D.; Modena, G. *Pure Appl. Chem.* **1982**, *54*, 1853–1866.

(13) Trudgill et al. (ref 7a) surveyed a range of cyclic and bicyclic ketones as substrates for this cyclohexanone oxygenase by spectrophotometric NADPH consumption assays. Only in the case of cyclohexanone was the product actually isolated and characterized (by infrared spectrophotometric and paper, thin-layer, and gas chromatographic comparison with authentic caprolactone).

(14) We thank Prof. David Ballou for suggesting iodide as a potential substrate for cyclohexanone oxygenase.

(15) Winnik, M. A.; Scott, V. *Can. J. Chem.* **1973**, *51*, 2788–2793.

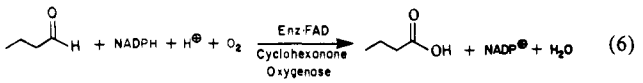
Baeyer–Villiger ketone oxygenations, except that hydrogen is the migrating group rather than carbon. Enzymatic processing of phenylacetaldehyde (Table I, eq 5) results in both hydrogen and benzyl migration (2:1 preference for hydrogen over benzyl migration as determined by ^{1}H NMR), as is also found in the reaction of phenylacetaldehyde with various peracids albeit with inverted migrating selectivity (7:1 to 2:1 preference for benzyl over hydrogen migration).[16] In contrast to the results with



(5)

phenylacetaldehyde, the oxygenation of aliphatic aldehydes with both peroxide-containing reagents and cyclohexanone oxygenase proceeds by essentially exclusive hydrogen migration to form the corresponding carboxylic acid.[17] In the enzymatic processing of the aliphatic aldehyde *n*-butyraldehyde, only *n*-butyric acid could be detected by ^{1}H NMR as a product of the reaction (Table I, eq 6).

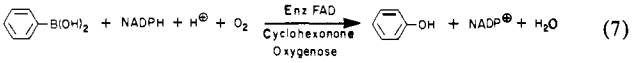
Significance of Migratory Aptitudes Observed in the Enzymatic Reactions. Cyclohexanone oxygenase performs aldehyde and ketone oxygenations with chemoselectivities similar to those observed in aldehyde and ketone oxygenations performed by peroxide-containing reagents. Since the migratory aptitudes in the



(6)

enzymic ketone and aldehyde oxygenations closely parallel those in model chemistry, *the enzyme must be performing a reaction with strong mechanistic similarities to the model chemistry* to allow the similar expression of such an intrinsic property of the substituent groups.

Deliver of Nucleophilic Oxygen: Oxygenation of Boronic Acid Substrates. The nucleophilic oxygen-transfer activity of cyclohexanone oxygenase toward the boronic acid functional group leads, after facile hydrolysis of the intermediate boronic acid ester (most likely nonenzymic hydrolysis), to the corresponding alcohol (Table I, eq 7 and 8). Boronic acids, well-known as reversible



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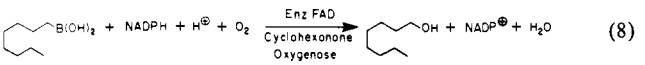


(8)

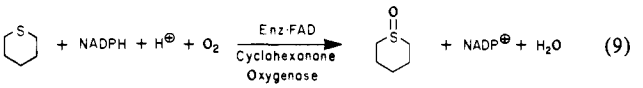
competitive inhibitors of serine proteases,[19] are aqueous-stable, enzymatically oxygenatable organoborane substrates.

Alkyl boronic acids are excellent substrates for oxygen transfer from cyclohexanone oxygenase compared to other substrates. For example, the *n*-octylboronic acid has a higher V_{max} and lower K_m than cyclohexanone. By the k_{cat}/K_m criterion, the *n*-octyl boronate is a very good substrate, with a value of 3.8×10^6 M^{-1} s^{-1}, only a few orders of magnitude away from the upper limit seen in enzymic catalysis.

The mechanism of boronic acid oxygenation reactions performed by peroxide-containing reagents 12a[18] is well established, yet somewhat obscure. Several recent reviews of reactions performed by peroxide-containing reagents completely overlooked the reaction.[12b-f]

Boronic acid oxygenation is an unprecedented activity for any flavoprotein monooxygenase. *To our knowledge boronic acid oxygenation has not been previously examined or characterized in any enzyme-mediated process.*

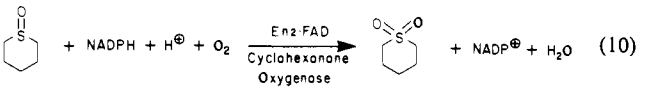
Enzymic Delivery of Electrophilic Oxygen to Heteroatom Substrates with Nucleophilic Lone Electron Pairs. The capability for cyclohexanone oxygenase to oxygenate the cyclic sulfide thiane to corresponding sulfoxide has been previously reported from our laboratory (Table II, eq 9).[7b] The enantioselective oxygenation



(9)

of ethyl *p*-tolyl sulfide to the corresponding sulfoxide by this cyclohexanone oxygenase was included in a survey from this laboratory of enantioselective sulfur oxygenations performed by various oxygenases (Table II).[7c] Sulfur oxygenation requires the delivery of a formally electrophilic oxygen atom equivalent to a formally nucleophilic sulfur atom lone pair of electrons.[12f] Various other heteroatoms possessing potentially nucleophilic electron lone pairs are known to undergo oxygenation with peroxide-containing reagents.[12]

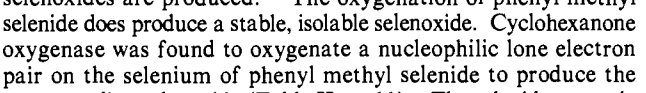
The lone nucleophilic electron pair on thianyl sulfoxide was found in turn to be readily oxygenated by cyclohexanone oxygenase to produce the corresponding sulfone (Table II, eq 10). The K_m



(10)

and V_{max} data for thiane and thianyl sulfoxide show that thiane is a better substrate for cyclohexanone oxygenase than is thianyl sulfoxide. This accounts for the fact that it is possible to perform an enzymatic incubation on thiane and isolate thianyl sulfoxide (along with unreacted thiane). Thianyl sulfoxide does not effectively compete with thiane for the enzymatic processing as long as thiane is present. Thianyl sulfoxide in the absence of thiane is efficiently processed to the corresponding sulfone. *These enzymatic results on reactivity of sulfides vs. sulfoxides can be directly related to the reactions of sulfides and sulfoxides with* peracids in which sulfides are more reactive than sulfoxides. For example, reaction of a sulfide with a peracid will cleanly produce the sulfoxide when 1 equiv of oxidizing reagent is used and the sulfone when 2 equiv is used.[12a,d]

Selenides are known to be readily oxygenated to the corresponding selenoxides with peracids and other peroxide-containing reagents.[20] In most instances the selenoxide products are too unstable to be isolated since they either undergo a facile β-elimination reaction if β-hydrogen atoms are present[20] or a facile 2,3-sigmatropic rearrangement reaction if allyl or propargyl selenoxides are produced.[20] The oxygenation of phenyl methyl selenide does produce a stable, isolable selenoxide. Cyclohexanone oxygenase was found to oxygenate a nucleophilic lone electron pair on the selenium of phenyl methyl selenide to produce the corresponding selenoxide (Table II, eq 11). The selenide enzymic



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(16) Rayer, M. J.; Beugelmans-Verrier, M. *C. R. Hebd. Seances Acad. Sci., Ser. C* **1971**, *272*, 1818–1820.

(17) (a) Hassall, C. H. *Org. React.* **1957**, *9*, 73–106. (b) Leading references on the reactions of aldehydes and ketones with hydrogen peroxide and alkyl hydroperoxides can be found in: "Comprehensive Organic Chemistry"; Barton, D., Ollis, W. D., Stoddard, J. F., Eds.; Pergamon Press, Fairview Park, Elmsford, NY, 1979; Vol. 1, pp 920, 936. The isolation of cyclic ketone–H_2O_2 tetrahedral adducts and other unrearranged oligomers: (c) Milas, N. A.; Harris, S. A.; Panagiotakos, P. C. *J. Am. Chem. Soc.* **1939**, *61*, 2430–2432. (d) Criegee, R.; Schnorrenberg, W.; Becke, J. *Annalen* **1949**, *565l*, 7–21. The reaction (in low yield) of acyclic ketones with H_2O_2: (e) House, H. O.; Wasson, R. L. *J. Org. Chem.* **1957**, *22*, 1157–1160. The efficient Baeyer–Villiger reaction of cyclic ketones with H_2O_2 at elevated temperatures: (f) Fox, S. W.; Polak, E. H.; Bullock, M. W.; Kobayashi, Y. *J. Am. Chem. Soc.* **1951**, *73*, 4979–4980. (g) Heine, H. W., Jones, H. *J. Am. Chem. Soc.* **1951**, *73*, 1361–1362. (h) Fling, M.; Minard, F. N.; Fox, S. W. *J. Am. Chem. Soc.* **1947**, *69*, 2466–2467. (i) Westerfeld, W. *J. Biol. Chem.* **1942**, *143*, 177–184. The isolation of aldehyde–H_2O_2 tetrahedral adducts and subsequent rearrangement by heating: (j) Spath, E.; Pailer, M.; Schmid, M.; *Chem. Ber.* **1941**, *74*, 1552–1556.

(18) (a) Kuivila, H. G. *J. Am. Chem. Soc.* **1954**, *76*, 870–874. (b) Kuivila, H. G.; Armour, A. G. *J. Am. Chem. Soc.* **1957**, *79*, 5659–5662. (c) Minato, H.; Ware, J. C.; Traylor, T. G. *J. Am. Chem. Soc.* **1963**, *85*, 3024–3026.

(19) For example see: Matteson, D. S.; Sadhu, K. M.; Lienhard, G. *J. Am. Chem. Soc.* **1981**, *103*, 5241–5242 and references therein.

(20) (a) Clive, D. L. J. *Tetrahedron* **1978**, *34*, 1049–1132. (b) Reich, H. J.; Yelm, K. E.; Wollowitz, S. *J. Am. Chem. Soc.* **1983**, *105*, 2503–2504. (c) Reich, H. J., et al. *J. Org. Chem.* **1975**, *40*, 2570–2572.

oxygenation is quite efficient with k_{cat}/K_m of 2×10^5 M^{-1} s^{-1}. Stereochemical questions were not addressed in this enzymatic oxygenation reaction since selenoxides are known to readily racemize in the presence of only traces of water via reversible formation of a tetracoordinate hydrate.[21,22]

Iodide is routinely used for the titrimetric determination of peroxides.[23-29] The reaction of iodide ion with peroxides requires the delivery of an electrophilic oxygen atom equivalent to a nucleophilic electron pair on the iodide ion. Iodide (sodium iodide) appeared by spectrophotometric NADPH consumption assays to be a reasonably good substrate for cyclohexanone oxygenase (Table II).

Summary and Discussion of Substrate Specificity and Catalytic Diversity of Cyclohexanone Oxygenase. With the single enzyme cyclohexanone oxygenase enzyme-catalyzed oxygenation, reactions significantly parallel the corresponding model reactions of substrate functional groups with peroxide-containing reagents in two distinct ways.

First, in analogy with peroxide-containing reagents, reactions can readily deliver either nucleophilic or electrophilic oxygen atom equivalents as required by the substrate. Second, in individual reactions in which more than one product is possible (phenylacetone, phenylacetaldehyde, *n*-butyraldehyde) or when relative reactivity trends can be observed (thiane vs. thiane sulfoxide), the enzyme-catalyzed reactions closely parallel the model chemistry performed by peroxide-containing reagents. In addition Schwab's studies on this cyclohexanone oxygenase also suggest a direct analogy between the enzyme and peracids.

We propose that the intermediate enzyme-bound 4a-F1-OOH in this cyclohexanone oxygenase[7b] *is the chemically competent oxygen-transfer species*; it would be expected to have chemical properties sufficient for and consistent with the broad spectrum of substrate oxygenations. All the available data on this cyclohexanone oxygenase do not require a more intricate explanation.

This minimal conceptualization of the enzymatic chemistry directly avoids speculation on the details of the enzymatic oxygen-transfer process from a 4a-F1-OOH. Even in simple nonenzymatic oxygenation reactions performed by peroxides, peracids, and other peroxide-containing reagents, it is not possible to formulate oxygen-transfer reactions in the intimate molecular detail[12e] inherent in the various possible mechanisms proposed for catalysis of oxygen transfer in flavoprotein monooxygenases.[9] A more detailed understanding of the oxygen-transfer reactions of peroxides, peracids, and other peroxide-containing reagents, particularly flavin hydroperoxide model compounds, should allow more meaningfully detailed mechanisms to be formulated for the cyclohexanone oxygenase and other flavoprotein monooxygenase reactions.

Potential Utility of Boronic Acids as General Probes for Nucleophilic Oxygen in Oxygenase Enzymes. The "natural" Baeyer–Villiger activity of cyclohexanone oxygenase requires the delivery of formally nucleophilic oxygen by the enzyme to the substrate. In contrast, the "natural" activity of many oxygenase enzymes, containing diverse cofactors such as flavins, pterins, copper, and iron-containing heme (cytochromes P-450), is the delivery of electrophilic oxygen atom equivalents.[2-5] Significant

Table III. Oxidizable Substrate

peroxide-containing oxidant	cyclohexanone	cyclohexyl methyl ketone	acetophenone	1,4-thioxane	*N,N*-dimethylbenzylamine	iodide	alkylboronic acids	arylboronic acids
tert-butyl hydroperoxide	slow[a]	slow[a]	slow[a]	5.8×10^{-5},[b] L/mol s	ca. 1×10^{-6},[b] L/mol s	1×10^{-3},[b] L/mol s		not quantitated but of practical utility[c]
hydrogen peroxide	slow[d]	slow[d]	slow[d]	1.4×10^{-4},[b] L/mol s	2.8×10^{-5},[b] L/mol s	1.0×10^{-3},[b] L/mol s	10^{-4} to 10^{-3},[e] L/mol s for five different compounds	1.6×10^{-2},[e] L/mol s for phenylboronic acid at pH 5.23
perbenzoic acid	96–158 $\times 10^{-5}$,[f,g] L/mol s ($CHCl_3$, 25 °C)	11.7–14.1 $\times 10^{-5}$,[h,i] L/mol s ($CHCl_3$, 25 °C)	1.5×10^{-5},[h] L/mol s ($CHCl_3$, 28 °C)				fast[j]	fast[j]
m-chloroperbenzoic acid				7.4×10^{2},[b] L/mol s	2.14×10^{3},[h] L/mol s	7.6×10^{2},[b] L/mol s	fast[j]	fast[j]
trifluoroperacetic acid	6.1×10^{-2},[k] L/mol s	5.7×10^{-3},[k] L/mol s (for isopropyl methyl ketone)	1.7×10^{-3},[k] L/mol s				fast[j]	fast[j]

[a] Alkyl hydroperoxides are not practical oxidants for Baeyer–Villiger oxidation reactins (ref 17). [b] Reference 8. [c] Reference 24. [d] Although Baeyer–Villiger oxidation can occur with hydrogen peroxide, the reaction is too slow to be a practical, general reaction (ref 17). [e] Reference 26. [f] Reference 25. [g] Reference 27. [h] Reference 28. [i] Reference 29 [j] Reference 30. [k] Reference 31.

(21) (a) For example, optically active phenyl methyl selenoxide can be completely racemized in less than 10 s by exposure to moisture: Davis, F. A.; Billmers, J. M.; Stringer, O. D. "Abstract of Papers", 186th American Chemical Society National Meeting, Washington DC, Aug 28–Sept 2, 1983; American Chemical Society: Washington, DC, 1983; ORGN 146. (b) Sharpless, K. B.; Young, M. W.; Lauer, R. F. *Tetrahedron Lett.* **1973**, 1979–1982 and references therein.

(22) May, S. W.; Phillips, R. S. *J. Am. Chem. Soc.* **1980**, *102*, 5983–5984.

(23) Siggia, S. "Quantitative Organic Analysis via Functional Groups", 4th ed.; Wiley-Interscience: New York, 1979; pp 325–342.

(24) Davies, A. G.; Moodie, R. B. *J. Chem. Soc.* **1958**, 2372–2377.

(25) Minato, H.; Ware, J. C.; Traylor, T. G. *J. Am. Chem. Soc.* **1963**, *85*, 3024–3026 and references therein.

(26) Mateos, J. L.; Menchaca, H. *J. Org. Chem.* **1964**, *29*, 2026–2028.

(27) Friess, S. L.; Frankenburg, P. E. *J. Am. Chem. Soc.* **1952**, *74*, 2679–2680.

(28) Friess, S. L.; Farnham, N. *J. Am. Chem. Soc.* **1950**, *70*, 5518–5521.

(29) Friess, S. L.; Pinson, R. *J. Am. Chem. Soc.* **1952**, *74*, 1302–1305.

mechanistic insight into the active oxygen-transfer species and oxygen-transfer steps in "naturally electrophilic" oxygenase enzymes might be gained if a general probe for the capability of oxygenase enzymes to deliver nucleophilic oxygen atom equivalents was available. The literature data collected in Table III demonstrated why the oxygenation of boronic acids may be useful as a general probe for nucleophilic oxygen in enzymes.

We have shown for cyclohexanone oxygenase that the sulfide functional group is a useful probe for electrophilic oxygen.[7b] The rates of oxidation of the sulfide (and amine and iodide) functional groups with various peroxide-containing reagents (Table III) suggests the general reactivity characteristics necessary for a useful general probe for electrophilic oxygen. Indeed, cytochrome P-450 oxygenases and the copper-containing dopamine β-monooxygenase are known to oxygenate sulfur-containing substrate analogues.[7c,22]

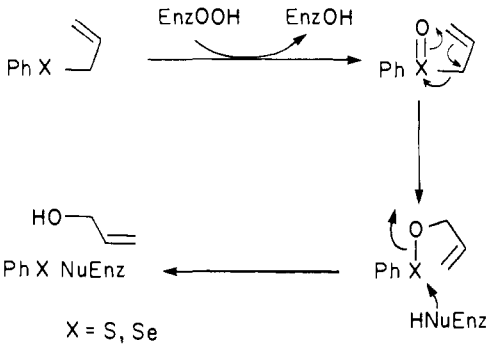
In general, the Baeyer–Villiger reaction[17a,b] can practically be performed in solution only by peracids. The rates of Baeyer–Villiger oxygenations performed by peracids are very slow compared to sulfide oxidation, even in the case of the highly reactive trifluoroperacetic acid (Table III). In general, the Baeyer–Villiger reaction of ketones with simple peroxides is not feasible because the readily formed, and often isolable, tetrahedral peroxy adducts of ketones with hydrogen peroxide[17c] or alkyl hydrogen peroxides are resistant to the alkyl migration step of the Baeyer–Villiger reaction.[17b-d] The tetrahedral peroxy adducts can react further to give peroxy adduct oligomers[17d] and can be forced to undergo Baeyer–Villiger migration upon heating.[17e-i] Thus, although the Baeyer–Villiger can occur with both peracids and peroxides under favorable conditions, it cannot be expected to be a reliable probe for nucleophilic oxygen in biological oxygenation reactions.

Although data are not shown in Table III for aldehydes as potential probes for nucleophilic oxygen, qualitative literature data suggest that the reactivity of aldehydes with peroxide-containing reagents directly parallels that of ketones. Aldehydes can be readily oxidized to the corresponding carboxylic acids with per acids in analogy to the Baeyer–Villiger reaction with peracids.[17a,b] Treatment of aldehydes with hydrogen peroxide generates stable and often isolable tetrahedral adducts which are resistant to further oxidative rearrangement,[17j] in analogy to the reactions of ketones with hydrogen peroxide or alkyl hydroperoxides. Therefore, aldehydes also cannot be expected to be general probes for nucleophilic oxygen.

Finally, the data on the oxygenation reactions of both alkyl and arylboronic acid (Table III) suggest that boronic acids should be useful as general probes for nucleophilic oxygen. First, the rates of boronic acid oxygenation with hydrogen peroxide are similar to the rate of sulfur oxygenation with hydrogen peroxide. Second, although quantitative data are not presented, it is known that per acid oxygenation of molecules containing carbon–boron bonds, including boronic acids, is much more facile than hydrogen peroxide oxygenation.[30,31] The reaction is so facile that peracid oxygen insertion into carbon–boron bonds is central to a general method for the quantitative microanalysis of boron in organic molecules.[30] Only limited data are available for peracid oxygenation of boron–carbon bonds because the reaction performed by peroxides is sufficiently facile for practical, synthetic purposes in which it has been used almost exclusively.[29]

From these quantitative and qualitative data, it is clear that boronic acids will readily react with essentially any peroxide-containing substance and that the oxygenation of boronic acids should be sufficiently facile to dependably act as a probe for nucleophilic oxygen. Also, it should be possible to incorporate aliphatic and aromatic boronic acid functional groups into almost any substrate analogue by the well-documented reaction of a Grignard reagent or an other carbanionic nucleophile with trimethyl borate followed by aqueous hydrolysis.[32] Probes for nucleophilic oxygen ranging from the simple methane boronic acid, $CH_3B(OH)_2$, up to any specific, complex boronic acid substrate analogue should be accessible.

Scheme IV



We believe that the use of such substrate analogues containing the boronic acid functional group may be useful probes for nucleophilic oxygen delivery capability in other oxygenase enzymes. Several other flavoprotein monooxygenases, such as mammalian sulfur–nitrogen oxygenases[5] and numerous bacterial phenolic aromatic hydroxylases,[4] are currently characterized in the literature as only exhibiting electrophilic oxygen deliver capability. The ability of these enzymes, which proceed through an enzyme-bound flavin hydroperoxide, to deliver nucleophilic oxygen has not been previously examined, due to the lack of a suitable probe for nucleophilic oxygen delivery capability.

The copper-containing monooxygenases and heme-containing monooxygenases (cytochromes P-450) are currently characterized as exhibiting only electrophilic oxygen deliver capability.[2,3] The availability of a new class of readily oxygenatable substrates requiring nucleophilic oxygen may provide some interesting and useful results with these enzymes.

Potential Utility of Cyclohexanone Oxygenase as a Reagent in Organic Synthesis. Cyclohexanone oxygenase has the potential to become important for enzyme-mediated organic oxygenation reactions. The properties of cyclohexanone oxygenase compare favorably in this regard with horse liver alcohol dehydrogenase (HLAD), the broad substrate specificity enzyme which has demonstrated synthetic utility for both enzyme-mediated reduction of ketones and aldehydes to alcohols and oxidation of alcohols to ketones and aldehydes.[33]

Pure cyclohexanone oxygenase is currently available in relatively large quantities (ca. 225 mg of pure enzyme from a bench-top 20-liter growth of cells; 310 g of wet-weight cell paste), and there is no reason that it could not be produced in multigram (or multikilogram) quantities if there were a genuine demand for it. The rate data for cyclohexanone oxygenase in the Baeyer–Villiger oxygenation of cyclohexanone to caprolactone (ca. 6 μmol/min mg at 15 °C; MW = 65 000 mg/mmol for the monomeric enzyme with one active site per monomer; turnover number = ca. 6–7 s^{-1}) compare favorably with that for HLAD in reduction of cyclohexanone to cyclohexanol (MW = 83 300 mg/mmol for the dimer of identical subunits with one active site per subunit; turnover number = ca. 18 and 9 s^{-1} average per active site).[34] The mechanistically informative but limited stereochemical data reported by Schwab[7d] and by our laboratory[7c] however suggest that

(30) For the reaction of boronic acids and other organoborane compounds with peracids: (a) ref 12a, pp 443–444 and references therein. (b) Pelter, A. In "Rearrangements in Ground and Excited States"; deMayo, P., Ed.; Academic Press: New York, 1980; Vol. 2, pp 110–116, particularly 114–115. (c) Strahm, R. D.; Hawthorne, M. F. *Anal. Chem.* **1960**, *32*, 530–531.

(31) Hawthorne, M. F.; Emmoms, W. D. *J. Am. Chem. Soc.* **1958**, *80*, 6398–6404.

(32) Pelter, A.; Smith, K. In "Comprehensive Organic Chemistry"; Barton, D., Ollis, W. D., Jones, D. N., Eds.; Pergamon Press: Fairview Park, Elmsford, NY, 1979; Vol. 3, pp 918–919 and references therein.

(33) (a) Jakovak, I. J.; Goodbrand, H. B.; Lok, K. P.; Jones, J. B. *J. Am. Chem. Soc.* **1982**, *104*, 4666–4671.

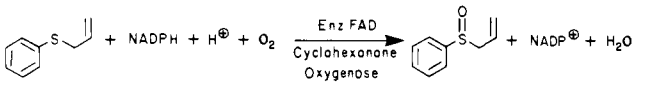
(34) Horse liver alcohol dehydrogenase activity was calculated by using specific activity = 78 μmol/min mg for acetaldehyde reduction and MW = 83 300 mg/mmol from: (a) Barman, T. E. "Enzyme Handbook"; Springer-Verlag: New York, 1969; Vol. 1, p 23, rate of cyclohexanone reduction = $^1/_6$ of rate of acetaldhyde reduction from: (b) "The Enzymes", 2nd ed.; Boyer, P. D., Lardy, H., Myrback, K., Eds.; Academic Press: New York, 1963; Vol. 7, p 40.

cyclohexanone oxygenase may be of limited utility as an enantioselective reagent in substrate oxygenation. The potential utility of cyclohexanone oxygenase as a chemoselective oxidant has not yet been reported although in 4-thiocyclohexanone, reaction flux appears to be exclusively by the Baeyer–Villiger mode.[35]

Allyl Sulfides and Allyl Selenides as Potential Mechanism-Based Inactivators for Cyclohexanone Oxygenase. This laboratory recently proposed a mechanism to account for an observed, apparent mechanism-based inactivation of a pyridoxal-linked enzyme which had as its key feature a 2,3-sigmatropic rearrangement of an allyl sulfoxide to generate an electrophilic allyl sulfenate at the enzyme's active site.[36] The enzyme-bound allyl sulfenate was proposed to be covalently captured by an enzyme active-site cysteine thiolate nucleophile, resulting in the formation of a covalent enzyme–inactivator adduct which blocked the enzyme's active site and thus inactivated the enzyme.

To assess the generality of the 2,3-sigmatropic rearrangement approach to the rational design of mechanism-based inactivators (Scheme IV) the processing of both phenyl allyl sulfide and phenyl allyl selenide by cyclohexanone oxygenase was examined. Both were found to be substrates for cyclohexanone oxygenase by spectrophotometric NADPH consumption assays (Table II).

For phenyl allyl sulfide the product phenyl allyl sulfoxide could be isolated and characterized by ^{1}H NMR and thin-layer chromatographic comparison with authentic phenyl allyl sulfoxide (Table II, eq 12), in accord with the known properties of phenyl



(12)

allyl sulfoxides.[20b,37] In continuous turnover spectrophotometric NADPH consumption assays with saturating concentrations of phenyl allyl sulfide present, no evidence could be found for significant turnover-dependent loss enzymatic activity for processing of this substrate for at least several hundred turnovers.

For phenyl allyl selenide by ^{1}H NMR, the extract of a preparative incubation did not contain the expected primary oxygenation product, phenyl allyl selenoxide, or the 2,3-sigmatropic rearrangement product, phenyl allyl selenate. These data are not unexpected in that rearrangement of phenyl allyl selenoxide is essentially instantaneous near room temperature (for phenyl allyl selenoxide $t_{1/2}$ at −40 °C = 60 min)[20c] and essentially irreversible (for the *o*-nitrophenyl allyl selenoxide–selenenate rearrangement, the selenenate is favored by approximately 11 kcal/mol at −80 °C).[20b] No attempt was made to isolate and characterize allyl alcohol, phenyl diselenide, and phenylselenic acid, the expected products of rearrangement, hydrolysis, and selenenic acid disproportionation.[38] Further studies on enzymatic oxygenation of various substituted and functionalized allyl and propargyl selenides in this laboratory have led to the isolation of products[35] which are fully consistent with the enzymatic oxygenation followed by rearrangement as proposed here for the prototype reaction. In numerous continuous turnover spectrophotometric NADPH consumption assays with saturating concentrations of phenyl allyl selenide present, no evidence could be found for significant turnover-dependent loss enzymatic activity from processing of this substrate for at least a thousand turnovers.

In the case of the allyl sulfoxide–allyl sulfenate 2,3-sigmatropic rearrangement, the rearrangement could be too slow to generate allyl sulfenate at the active site before product release. Given a k_{cat} of ca. 5 s^{-1} at 15 °C, it may well be that rearrangement of product only occurs off the enzyme. Since the allyl selenoxide–allyl selenate rearrangement essentially irreversibly generates the allyl selenate approximately 10^6 times faster than the corresponding allyl sulfoxide–allyl sulfenate rearrangement in organic solvents,[20b] it becomes difficult to rationalize the essentially identical insensitivity of cyclohexanone oxygenase to turnover of both phenyl allyl sulfide and phenyl allyl selenide as being mainly due to product release prior to rearrangement.

Although the 2,3-sigmatropic rearrangement strategy for mechanism-based inactivation has so far failed in the case of cyclohexanone oxygenase, demonstration of enzymatic oxygenation of allyl selenides to allyl selenoxides capable of extraordinarily facile 2,3-sigmatropic rearrangement may suggest a viable strategy for the inactivation of other enzymes.

Conclusion

The spectrum of monooxygenations performed by cyclohexanone oxygenase is now shown to be much wider than that performed by any other single flavoprotein monooxygenase. Cyclohexanone oxygenase may not be unique in having such a broad spectrum of activity since other flavoprotein monooxygenases may also possess oxygenation activities which have not as yet been searched for. Considering the different requirements that the various substrates have in order to be efficiently oxygenated, the simplest interpretation of these data is that the flavin 4a-hydroperoxide intermediate in the catalytic cycle is the active oxygen-transfer species.

Boronic acid substrate analogues were found to be readily oxygenated by cyclohexanone oxygenase in the first example of an enzyme-mediated boronic acid oxygenation. It is likely that boronic acids may become general probes for nucleophilic oxygen delivery capability in oxygenase enzymes presently characterized in the literature as delivering electrophilic oxygen, such as various monooxygenases utilizing pterin, copper, and iron-containing heme (cytochromes P-450) cofactors. (In the P-450 case it is unlikely that the $Fe^{V}O$ species will possess nucleophilic oxygen delivery capability but the precursor $Fe^{III}OOH$ might possess that capability.)

Due to its ready availability in large quantities and its broad substrate specificity, cyclohexanone oxygenase may be useful as an enantioselective and/or chemoselective oxidant in preparative organic chemistry, although further study is needed to fully assess its utility as a chemical reagent.

Attempted mechanism-based inactivation of cyclohexanone oxygenase utilizing a 2,3-sigmatropic rearrangement strategy with phenyl allyl sulfide and phenyl allyl selenide failed. The strategy of using the very facile 2,3-sigmatropic rearrangement of allyl selenoxides to generate electrophilic allyl selenenates may be of utility in the design of mechanism-based inactivators for other enzymes.

Experimental Section

Bacterial Growth. A culture of *Acinetobacter* NCIB 9871, maintained in this laboratory from a culture generously donated by Dr. P.W. Trudgill of the University College of Wales at Aberystwyth, United Kingdom, was used to produce cyclohexanone oxygenase. Our protocol for growth of the bacteria is conceptually similar to the previously reported protocol[7] but differs in several specific technical aspects which allow it to be performed on any laboratory bench-top with essentially no specialized equipment. Cultures were grown under standard sterile conditions (autoclaving of equipment and bulk solutions and sterile filtration of concentrated salt solutions added to make up final medium) in a 5-L glass carboy maintained at 30 °C in a standard thermostated water bath. The final 20 L of minimal media contained $KHPO_4$ (2.0 g/L), Na_2PO_4 (4.0 g/L), $(NH_4)_2SO_4$ (3.0 g/L, $MgSO_4$–$7H_2O$ (0.5 g/L), $FeSO_4$–$7H_2O$ (0.01 g/L), $CaCl_2$–$2H_2O$ (0.11 g/L), and Difco No. 0127.01 yeast extract (1.0 g/L) in distilled water. The fermentation was vigorously oxygenated with a pure O_2 source delivered through a fritted glass gas dispersion inlet. Bacterial production of cyclohexanone oxygenase was induced by the periodic addition (every few hours at outset then more often near the end of growth) of cyclohexanol to the fermentation until 0.5 g/L of cyclohexanol was added regularly. The pH was periodically adjusted (every few hours) with 50% NaOH to approximately pH 8.2 since the bacteria produced acid, and the growth of these bacteria is known to stop below pH 6.5. Bacterial growth was monitored with a

(35) Latham, J.; Walsh, C. unpublished results.
(36) Johnston, M.; Raines, R.; Walsh, C.; Firestone, R. *J. Am. Chem. Soc.* **1980**, *102*, 4241–4250.
(37) (a) Evans, D. A.; Andrews, G. C. *Acc. Chem. Res.* **1974**, *7*, 147–155. (b) Bickart, P.; Carson, F. W.; Jacobus, J.; Miller, E. G.; Mislow, K. *J. Am. Chem. Soc.* **1968**, *90*, 4869–4876. (c) Tang, R.; Mislow, K. *Ibid.* **1970**, *92*, 2100–2104.
(38) (a) Reference 20a, p 1107. (b) Hori, T.; Sharpless, K. B. *J. Org. Chem.* **1978**, *43*, 1689–1697. (c) Reich, H. J.; Wollowitz, S.; Trend, J. E.; Chow, F.; Wendelborn, D. F. *J. Org. Chem.* **1978**, *43*, 1697–1705.

Klett meter, and bacteria were harvested by centrifugation in the late logarithmic growth phase. The wet cell paste was frozen in liquid nitrogen and stored at −70 °C.

Isolation and Purification of Cyclohexanone Oxygenase. We have developed an improved preparation of cyclohexanone oxygenase which is homogeneous by sodium dodecyl sulfate poly(acrylamide) discontinuous gel electrophoresis. The new preparation is based on the original preparation developed by Trudgill[7a] which was modified by Ryerson in this laboratory.[7b] It involves rupture of the cells with a French pressure cell, ammonium sulfate fractionation, DE-52 ion-exchange chromatography, and Amicon Matrex Red Dye–Ligand affinity chromatography (elution of column-bound enzyme with NADP). The progress of the purification was monitored by spectral ratio determinations of flavin content per protein (A_{280}/A_{440} = 9.0 for the homogeneous enzyme) and by 4-methylcyclohexanone stimulated NADPH consumption spectrophotometric (A_{340}) assays (final specific activity = 6.1 Units/mg). Full details of this preparation will be reported in the near future.

Analytical Instrumentation. ^{1}H NMR spectra were obtained on Varian T-60, Bruker 250, or Bruker 270 instruments. Chemical shifts are reported in δ relative to the internal standard Me_4Si for CW NMR spectra obtained at 60 MHz and internal standard $CHCl_3$ impurity in $CDCl_3$ for FT NMR spectra obtained at 250 and 270 MHz. Mass spectra were measured on a Varian MAT 44 instrument at MIT or an AEI MS-9 instrument at Harvard University, Department of Chemistry (kindly performed by David Hansen). Melting points were determined with a Mel-Temp apparatus and are uncorrected.

General Experiment for Preparation of Substrates and Authentic Samples of Products and Analysis of Enzymatic Incubation Products. Reagent grade solvents were used. Aldrich Gold Label $CDCl_3$ was used for ^{1}H NMR spectroscopy. Merck No. 5765-7 Silica Gel 60 F-254 analytical plates were used for all thin-layer chromatography analyses.

Substrates and Authentic Samples of Products. The substrates and products employed were either commercially available or were prepared according to literature preparations with minor variations. The identity of these simple compounds was evident from the method of preparation and characteristic ^{1}H NMR spectral data. Commercially obtained compounds were assessed to be sufficiently pure to be used as received or were further purified as noted in the following paragraph. The minimum purity of all substrates used in enzymatic assays was estimated by ^{1}H NMR to be ca. 95% and by silica gel TLC analysis to be ca. 100% (single spot using various standard visualization techniques; UV-visible, phosphomolybdic acid/EtOH, iodine vapors, *p*-anisaldehyde-H_2SO_4/EtOH).

Substrates. **Phenylacetone** was used as received. **Phenylacetaldehyde** was distilled at 1 atm under argon prior to use. ***n*-Butyraldehyde** was distilled at 1 atm under argon prior to use. **Phenylboronic acid** was used as received. ***n*-Butylboronic acid** was used as received. ***n*-Octylboronic acid** was prepared by the method of Snyder et al.[39a] and recrystallized from CH_3NO_2; the melting point was not taken since such data are known to be highly variable and not an indicator of purity of boronic acids:[39b] ^{1}H NMR ($CDCl_3$) δ 4.27 (s, 2 H), 1.5–1.2 (m, 12 H), 0.95–0.75 (m, 5 H); mass spectrum (with heating), m/z 420 (trimer anhydride), (without heating) m/z 140 (monomeric anhydride). **Thiane sulfoxide** was prepared by $NaIO_4$ oxidation of thiane in methanol–water according to the method of Carlson and Helquist.[40] The white deliquesecent solid which was obtained by 1:1 CH_2Cl_2/Et_2O extraction of the residue obtained from evaporation of the crude reaction was found to be of sufficient purity for use in enzymatic incubations. ^{1}H NMR ($CDCl_3$) δ 3.0–2.7 (m, 4 H), 2.35–2.15 (m, 2 H), 1.75–1.55 (m, 4 H). **Thiane sulfone** was prepared by $KMnO_4$/acetone oxidation of thiane followed by silica gel chromatographic purification: mp 100.5–102 °C after evaporation from EtOH solution (lit. mp 98.5–99 °C crystallized from H_2O);[41] ^{1}H NMR ($CDCl_3$) δ 3.05–2.85 (m, 4 H), 2.20–1.95 (m, 4 H), 1.70–1.55 (m, 2 H). **Phenyl allyl selenide** was prepared according to the method of Binns and Haynes.[42] The crude product was distilled under argon under water aspirator vacuum prior to use: ^{1}H NMR ($CDCl_3$) δ 7.6–7.5 (m, 2 H), 7.4–7.3 (m, 3 H), 6.1–5.95 (m, 1 H), 5.1–4.95 (m, 2 H), 3.59 (d, J = ca. 7 Hz, 2 H). **Phenyl methyl selenide** was prepared by the general method of Binns and Haynes.[42] The crude product was distilled under argon under water aspirator vacuum prior to use: ^{1}H NMR ($CDCl_3$ δ 7.5–7.4 (d, J = 6.7 Hz, 2 H), 7.35–7.15 (m, 3 H), 2.36 (s, 3 H). **Phenyl allyl sulfide** was prepared according to the method of Binns and Haynes[42] for the preparation of phenyl allyl selenide but performed at room temperature rather than at 0 °C. The crude product was distilled under argon under water aspirator vacuum prior to use: ^{1}H NMR ($CDCl_3$) δ 7.5–7.2 (m, 5 H), 6.05–5.85 (m, 1 H), 5.19 (d, J = 17.1 Hz) and 5.13 (d, J = 9.8 Hz, 2 H total), 3.60 (d, J = 6.8 Hz, 2 H).

Products. **Phenylacetic acid** was used as received. **Benzyl formate** was used as received. **Benzyl alcohol** was used as received. ***n*-Butyric acid** was used as received. **Phenol** was used as received. ***n*-Octyl alcohol** was used as received. **Phenyl methyl selenoxide** was prepared by $NaIO_4$/methanol–water oxidation following the general procedure of Carlson and Helquist for the oxidation of sulfides to sulfoxides.[40] The crude product was distilled under argon under water aspirator vacuum prior to use: ^{1}H NMR ($CDCl_3$) δ 7.85–7.7 (m, 2 H), 7.65–7.5 (m, 3 H), 2.61 (s, 3 H). **Phenyl allyl sulfoxide** was prepared by $NaIO_4$/methanol–water oxidation following the general procedure of Carlson and Helquist.[40] The crude product was distilled under argon under water aspirator vacuum prior to use: ^{1}H NMR ($CDCl_3$) δ 7.6–7.5 (m, 2 H), 7.5–7.4 (m, 3 H), 5.7–5.5 (m, 1 H), 5.27 (d, J = 9.8 Hz), 5.14 (d, J = 16.6 Hz, 2 H total), 3.6–3.4 (m, 2 H).

Kinetic Assays. K_m and V_{max} were determined from Lineweaver–Burk plots of 1-mL spectrophotometric assays of enzyme activity (ca. 0.1 μM enzyme concentration) in 80 mM pH 9.0 glycine–NaOH monitoring NADPH consumption (NADPH concentration = ca. 200 μM corresponding to an absorbance of ca. 1 through a 1-cm path length) at 340 nm stimulated by the addition of 1–20 μL of a methanolic solution of substrate. Assays were conducted at 15 °C because incubation of the enzyme in the presence or absence of NADPH and in the absence of substrate at 30 °C, the temperature previously reported for assays,[7] resulted in the gradual loss of enzymatic activity, apparently by thermal denaturation. The enzyme is much more stable at 15 °C in the absence or presence of NADPH and/or substrates.

Product Isolation and Characterization Studies. Large-scale incubations were set up in 125-mL Erlenmeyer flasks equipped with a stirring bar and a large ice–water cooling bath. A control reaction (with no enzyme) was set up and run simultaneously in the same cooling bath except for one case noted in Table I.

The incubations contained pH 9.0 80 mM glycine–NaOH (50 mL), substrate (20–50) μmol of substrate added as a solution in 0.500 mL of methanol), Sigma No. N 6505 β-nicotinamide adenine dinucleotide reduced form tetrasodium salt [NADPH] (20–30 mg, 24–36 μmol), pure cyclohexanone oxygenase (2–10 nmol from various samples with ca. 1.5–4.9 μmol/min mg activity with cyclohexanone at 15 °C in this buffer [activities vary with age and specific history of various samples]; the enzyme was added as 0.100–0.500 mL of solutions in standard preparation storage buffer, pH 9.0, 80 mM glycine–NaOH: 5 mM Sigma D 9760 dithiothreitol), and in several cases Sigma C-100 bovine liver catalase 2X crystallized (0.010–0.020 mL of 45 mg/mL of suspension which was found to have 15 000 μmol/min mg activity in pH 9.0, 80 mM glycine–NaOH at 15 °C using a minor modification of the standard Sigma catalase assay). Control reactions were prepared identically at the same time except that enzyme was omitted. Both incubations for each determination were set up by weighing NADPH into the Erlenmeyer flasks followed by the addition of buffer. Methanolic solutions of substrates were added to the incubation solutions and then they were cooled with stirring to 10–15 °C over 5–10 min. Catalase (if used) was then added to both incubations followed by the addition of cyclohexanone oxygenase solution to only one of the two incubations. Incubations were then stirred (to ensure adequate replenishment of O_2 in solution) for 1–2 h at 10–15 °C. Incubations were terminated by acidification to pH 1 with concentrated HCl followed by extraction with CH_2Cl_2 or Et_2O or by direct extraction without acidification. Analysis of the residue obtained by evaporation of the organic extracts for oxygenation products in the cyclohexanone oxygenase incubations and their absence in the control incubations was by ^{1}H NMR (Bruker 250 or 270) comparison with authentic compounds and in several instances by silica gel TLC comparison with authentic compounds as noted in Tables I and II. A representative incubation with phenylboronic acid is detailed in the following paragraph.

Incubation of Phenylboronic Acid with Cyclohexanone Oxygenase. As described in the general experimental, a product-generating incubation was set up containing phenylboronic acid (0.500 mL of a 48.8 mM solution in methanol, 2.98 mg, 0.0244 mmol), NADPH (13 mg, 0.0156 mmol), catalase (0.010 mL of 45 mg/mL of enzyme with 15 000 μmol/min mg, 0.45 mg of enzyme, 6750 μmol/min mg of catalase activity), and cyclohexanone oxygenase (0.100 mL of 3.354 mg/mL of stock solution containing 4.93 μmol/min mg of cyclohexanone activity at 15 °C in the incubation buffer, 0.335 mg of enzyme, 1.65 μmol/min mg of cyclohexanone acticity) in 50 mL of pH 9.00, 80 mM glycine–NaOH buffer. An identical control reaction without cyclohexanone

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oxygenase was set up simultaneously.

After 1 h of stirring at 10–15 °C, both the product-generating and control incubations were acidified to pH 1 (by pH paper) with concentrated HCl. Each incubation was extracted with 4 × 50 mL of CH_2Cl_2. The organic extracts were combined and then filtered through cotton (to remove water). After rotary evaporation at water aspirator vacuum, residual solvents were removed on a vacuum line. The crude residues obtained in this manner from each incubation were analyzed by high-field ^1H NMR for the presence of phenylboronic acid starting material and the presence or absence of phenol. In ^1H NMR of the control, of the two substances of interest, only phenyl boronic acid and not a trace of phenol could be detected. On the other hand, in the ^1H NMR of the product-generating incubation, both phenylboronic acid and phenol could be detected in the ratio phenylboronic acid/phenol = 3.2:1.0. Silica gel TLC (25% ethyl acetate/benzene; visualization with ultraviolet light, phosphomolybdic acid/EtOH, *p*-anisaldehyde–H_2SO_4/EtOH) confirmed these results for the presence of phenylboronic acid (R_f = 0.36) and the presence or absence of phenol (R_f = 0.75).

Registry No. Cyclohexanone oxygenase, 52037-90-8; cyclohexanone, 108-94-1; phenylacetone, 103-79-7; 2-phenyl-1-ethanal, 122-78-1; butanal, 123-72-8; phenylboronic acid, 98-80-6; *n*-butylboronic acid, 4426-47-5; *n*-octylboronic acid, 28741-08-4; thiane, 1613-51-0; ethyl *p*-tolyl sulfide, 622-63-9; phenyl allyl sulfide, 5296-64-0; thiane sulfoxide, 4988-34-5; phenyl methyl selenide, 4346-64-9; phenyl allyl selenide, 14370-82-2; triethyl phosphite, 122-52-1; sodium iodide, 7681-82-5; *tert*-butyl hydroperoxide, 75-91-2; hydrogen peroxide, 7722-84-1; perbenzoic acid, 93-59-4; *m*-chloroperbenzoic acid, 937-14-4; trifluoroperacetic acid, 359-48-8; cyclohexyl methyl ketone, 823-76-7; acetophenone, 98-86-2; 1,4-thioxane, 15980-15-1; *N*,*N*-dimethylbenzylamine, 103-83-3.

Aggregate Morphology and Intermembrane Interaction of Synthetic Peptide Lipids Bearing Various Head Groups

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Abstract: Peptide lipids bearing three different head groups, cationic $N^+C_5Ala2C_n$, anionic $(SO_3^-)C_5Ala2C_n$, and nonionic QC_5Ala2C_n, were prepared for the investigation of their aggregate morphology and intermembrane interaction between the ionic and nonionic lipid aggregates in aqueous media. Anionic lipids bearing the sulfonate head group, $(SO_3^-)C_5Ala2C_n$, form primarily lamellar aggregates in the aqueous dispersion state, which are converted into double-walled bilayer vesicles upon sonication. In contrast with the ionic lipids, nonionic lipids bearing the quinoyl head group, QC_5Ala2C_n, form highly ordered network assemblies in the dispersion state in the neutral pH range, and the sonication of such aggregates afforded scattered cloudlike assemblies without formation of small bilayer vesicles. QC_5Ala2C_n behaves as a normal anionic lipid under conditions that the hydroxyl groups of the quinoyl moiety are deprotonated and forms multiwalled bilayer vesicles in the dispersion state. The morphological change of the nonlamellar network aggregates of QC_5Ala2C_n is induced by intermembrane interaction with ionic peptide lipids to afford the normal bilayer membranes. The present results obtained by turbidity and differential scanning calorimetry measurements strongly indicate that the morphological change is induced by the one-way intermembrane transfer of the ionic lipid molecules through the intervening aqueous phase and the rate-determining step is the diffusion of the ionic lipid molecules from the bilayer phase to the bulk aqueous phase.

Clarification of aggregate morphology of synthetic lipids and characterization of their intermolecular and intervesicular interactions in aqueous media are indispensable not only for understanding physicochemical functions of biological membranes but also for developing membrane-mimetic reaction media. To overcome complexities and chemical instabilities of naturally occurring membranes, a large number of membrane-forming lipids have been prepared.[1] Most of these synthetic lipids as well as natural ones tend to form thermodynamically stable bilayer aggregates. Meanwhile, nonbilayer aggregates such as the inverted hexagonal (H_{II}) phase are also found under certain conditions in natural systems, and these nonbilayer phases may well be involved in dynamic intermembrane interactions.[2] As regards model membranes, however, there are few studies to clarify the intrinsic factors which control the formation of bilayer and nonbilayer aggregates and their interconversions due to the lack of appropriate synthetic lipids. We have recently prepared a series of peptide lipids involving an amino acid residue interposed between a polar head moiety and an aliphatic double-chain segment.[3] The lipids are so designed as to constitute the hydrogen-belt domain upon formation of bilayer aggregates in the light of the concept, so-called tripartite structure having a hydrogen-belt domain, for biomembranes.[4] The cationic and zwitterionic peptide lipids have been observed to form bilayer membranes (vesicles and/or lamellae) in the aqueous dispersion state which are subsequently converted into distinct single-compartment vesicles upon sonication.[3] Such single-compartment vesicles once formed remain in the solution without significant morphological change for a reasonably prolonged period of time. In contrast, a nonionic lipid having the quinoyl group as its polar head forms nonlamellar network aggregates[5] which undergo morphological change as induced by intermembrane interaction with cationic bilayer vesicles.[6]

In the present work, we have studied primarily on the following two subjects. Firstly, the correlation between the nature of head groups of lipids and the aggregate morphology is clarified by

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