Pyrimidine Ring Contraction with a 5-Deazaflavin 4a,5-Epoxide Derivative. Model Studies for Reactions with Enzyme-Bound Epoxide Intermediates

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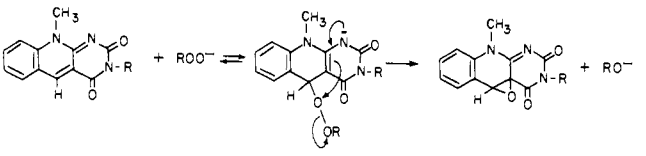
Abstract: 5-Deazaisoalloxazine 4a,5-epoxide has been converted to 4-methyloxazolo[4,5-*b*]quinolin-2(4*H*)-one, a purple fluorescent compound that exhibits properties similar to a chromophore formed with enzyme-bound 5-deazaflavin 4a,5-epoxide at neutral pH. The model reaction proceeds via two spectrally detectable intermediates (A, B). Alkaline conditions are required only for conversion of the epoxide to intermediate A. It is proposed that intermediate A is a glycidic acid derivative formed via hydrolysis of the pyrimidine ring in the parent epoxide. Ionization of intermediate A (pK_a = 3.00) causes an 11-fold decrease in the rate of its conversion to intermediate B. This step is postulated to involve decarboxylation and epoxide ring opening. This generates an enolic hydroxyl group, which then attacks the carbonyl carbon in the carbamoylimino function, causing ring closure and release of RNH_2. The last step (reversible with excess RNH_2) is rate determining at pH >12 while conversion of intermediate A to intermediate B is the slow step in reactions initiated with intermediate A at pH 2.8-11.1.

The discovery of 5-deazaflavin as a naturally occurring cofactor in various bacteria and its use as an artificial coenzyme in mechanism studies with numerous flavoproteins[1] has stimulated considerable interest in the chemistry of these modified flavins. While hydroperoxide derivatives are formed with normal flavin and H_2O_2, 5-deazaisoalloxazine reacts with either H_2O_2 or *m*-chloroperoxybenzoic acid to form a novel 4a,5-epoxide derivative.[2] Recent kinetic studies in aqueous solution show that epoxidation with either reagent involves peroxide anion as the reactive species[3] (Scheme I). The results with *m*-chloroperoxybenzoic acid appear to provide the first direct evidence for epoxidation with an ionized peroxy acid.[4] An analogous epoxide derivative has been identified as an intermediate in the reaction of peroxides with 5-deazaflavin bound to several flavoproteins.[1] Unlike 5-deazaisoalloxazine 4a,5-epoxide, which slowly decays in neutral aqueous solution, enzyme-bound epoxide is converted to a stable purple fluorescent chromophore, deazaflavin-X, in a reaction facilitated by the protein moiety.[1] In this communication we report a reaction initiated by exposure of 5-deazaisoalloxazine 4a,5-epoxide to alkaline conditions that appears analogous to the reaction observed with enzyme-bound epoxide.

Scheme I



Experimental Section

The synthesis of compound C (Scheme II) with 3,10-dimethyl-5-deazaisoalloxazine 4a,5-epoxide was initiated by mixing the epoxide[2] (200 mg, 0.78 mmol in 40 mL of Me_2SO) with 360 mL of 1.0 mM sodium phosphate pH 11.1 for 1 min. The solution was then diluted with 1600 mL of 0.12 M sodium pyrosphosphate, pH 7.5. After 90 min at 25 °C the product was extracted with chloroform. The solvent was evaporated and the residue recrystallized several times from chloroform-ether. For analysis preparative thin layer chromatography (silica gel 60, chloroform-1-butanol (7:3) as solvent) was performed (35% yield, mp 245-247 °C); ^{1}H NMR ($CDCl_3$) δ 4.15 (s, 3 H, N(4) CH_3), 7.41 (s, 1 H, C(9) H), 7.47-7.80 (m, 4 H, Ar H), no exchangeable protons. Anal. Calcd for $C_{11}H_8N_2O_2$: C, 66.00; H, 4.03; N, 13.99. Found: C, 65.63; H, 4.11; N, 13.97. The same procedure was used to prepare compound C from 10-methyl-5-deazaisoalloxazine 4a,5-epoxide.[2] In addition to visible, ^{1}H NMR, and mass spectral data, the identity of the products prepared from different epoxides was verified by mixture melting point experiments and thin layer chromatography (silica gel 60 F-254, chloroform-1-butanol (7:3), benzene-ethanol (7:3), or chloroform-ethanol (4:1) as solvent).

Studies to show that methylamine is formed during the reaction with 3,10-dimethyl-5-deazaisoalloxazine 4a,5-epoxide were conducted by using the aqueous phase remaining after chloroform extraction of compound C. The solution was made alkaline, and methylamine was isolated by refluxing for 2 h in a flask fitted with a water condenser connected to a trap containing 6 N HCl. The HCl solution was then evaporated and the residue reacted with *p*-toluenesulfonyl chloride.[5a] The *N*-methyl-*p*-toluenesulfonamide product was recrystallized from hot ethanol (yield 19%, mp 74.5-75 °C (lit. mp 75 °C[5b]). Product identity was verified in a mixed melting point experiment with a known standard.

In studies to identify CO_2 as a reaction product, aqueous solutions were prepared with boiled deionized water under an atmosphere of CO_2-free argon, which was maintained throughout the experiment. The argon was scrubbed with a column containing alternate layers of solid KOH and $Ba(OH)_2$, followed by a saturated solution of $Ba(OH)_2$. 3,10-Dimethyl-5-deazaisoalloxazine 4a,5-epoxide (175.8 mg, 0.68 mmol in 40 mL of Me_2SO) was mixed with 360 mL of 1.0 mM sodium phosphate, pH 11.3, at 25 °C for 2 min and then acidified to pH 4 with HCl. Argon was bubbled through the solution and then through two tubes each containing 10 mL of saturated $Ba(OH)_2$. After 3.5 h the CO_2 traps were stoppered and centrifuged. The $BaCO_3$ precipitate was washed with 2-propanol and then with ether and dried (yield 119.2 mg, 88.3%). No $BaCO_3$ was detected in a control lacking epoxide.

All kinetic studies were conducted at 25 °C. At pH ≤11.1 the conversion of intermediate A to compound C was monitored at 330 nm. Values for the rate of conversion of intermediate A to intermediate B (k_1) were determined from linear first-order plots of absorbance changes at this wavelength. Reactions at pH 2.8-9.2 were initiated with intermediate A (2×10^{-5} M), prepared by a short preincubation (45 s) of

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(1) Vargo, D.; Pokora, A.; Wang, S.; Jorns, M. S. *J. Biol. Chem.* **1981**, *256*, 6027-6033 and references therein.

(2) Vargo, D.; Jorns, M. S. *J. Am. Chem. Soc.* **1979**, *101*, 7623-7626 and references therein.

(3) Vargo, D. Ph.D. Dissertation, The Ohio State University, 1981.

(4) (a) Kinetic parameters for the epoxidation of 3,10-dimethyl-5-deazaisoalloxazine with *m*-chloroperoxybenzoate (k = 1360 M^{-1} s^{-1}, pK_a = 7.63, lit. pK_a = 7.60[4b]) were evaluated at 25 °C. Indirect evidence for peroxycarbonate as an epoxidizing agent has been reported.[2] As compared with alkaline epoxidation with ROO^- (R = H, alkyl),[2] reaction with $RC(O)OO^-$ in neutral aqueous solution may provide a gentle alternative method for other compounds containing a carbon double bond conjugated to unsaturated electron-withdrawing groups that exhibit decreased reactivity towards epoxidation with unionized peroxy acids.[4c] (b) Curci, R.; Edwards, J. O. In "Organic Peroxides"; Swern, D., Ed.; Wiley-Interscience: New York, 1970; Vol. 1, pp 199-264. (c) Swern, D. In "Organic Peroxides"; Swern, D., Ed.; Wiley-Interscience: New York, 1971; Vol. 2, pp 355-534.

(5) (a) Vogel, A. "Textbook of Practical Organic Chemistry"; Longman: London, 1979; p 1130. (b) Shriner, R. L.; Fuson, R. C.; Curtin, D. Y. "The Systematic Identification of Organic Compounds"; Wiley: New York, 1956; p 326.

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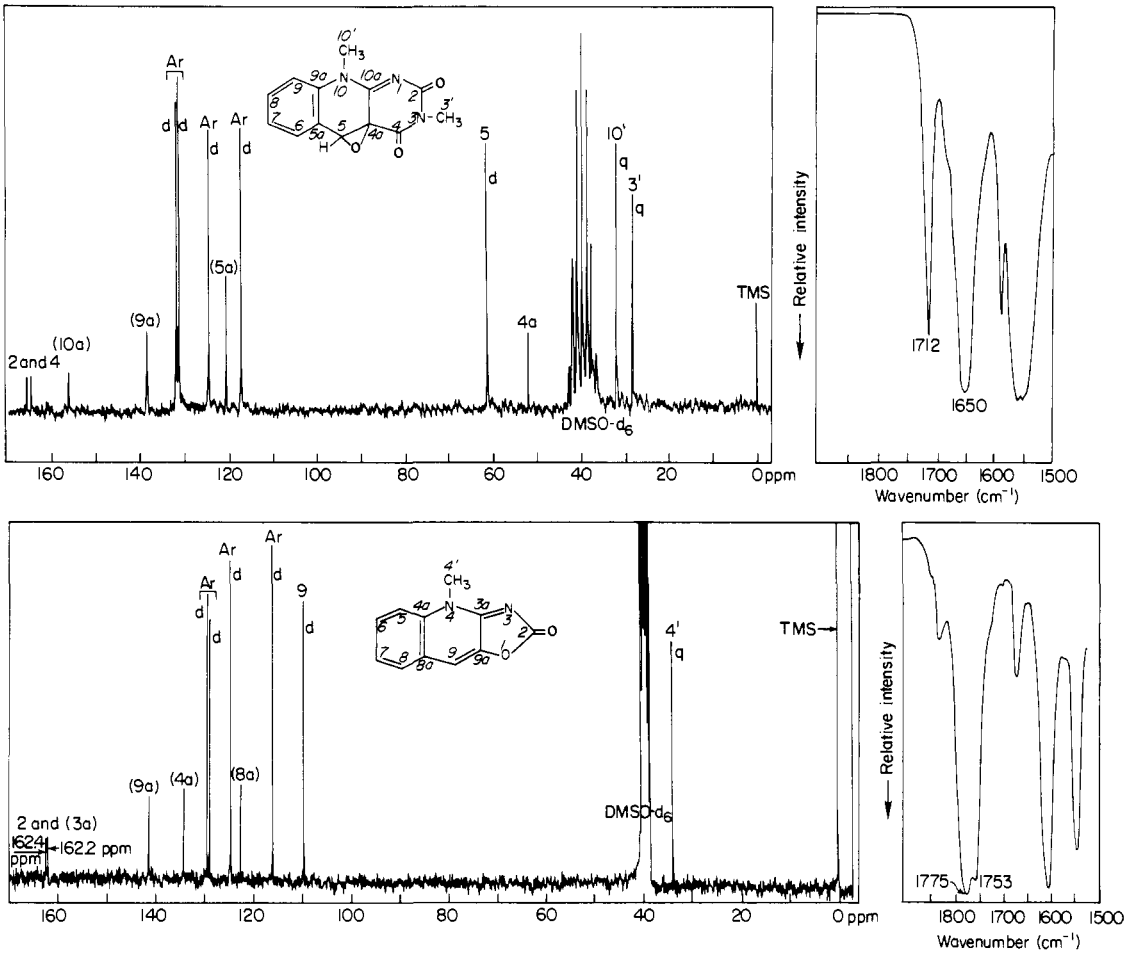
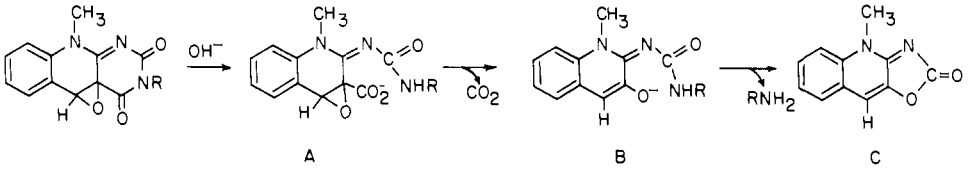


Figure 1. Wide-band ^{1}H-decoupled ^{13}C NMR (in Me_2-d_6-SO) and infrared (KBr pellet) spectra of 3,10-dimethyl-5-deazaisoalloxazine 4a,5-epoxide and compound C. Unless otherwise indicated, singlets were observed in off-resonance ^{1}H-decoupled experiments. Peak assignments for the methyl groups in the epoxide are based on previous studies.[8] Assignments shown in parentheses are tentative.

Scheme II



3,10-dimethyl-5-deazaisoalloxazine 4a,5-epoxide (8×10^{-5} M) with 1.0 mM sodium phosphate, pH 11.1. At pH >12 values for k_1 and for the rate of conversion of intermediate B to compound C (k_2) were determined by analysis[6] of absorbance changes at 375 nm, a wavelength where only intermediate B absorbs. In other experiments the conversion of intermediate B to compound C was monitored at neutral pH (0.1 M sodium pyrophosphate plus 0.2 mM sodium phosphate, pH 7.7) with intermediate B prepared by reacting 3,10-dimethyl-5-deazaisoalloxazine 4a,5-epoxide with 1 mM sodium phosphate, pH 12.2, for 11 or 36 min. Similar results were obtained for the reaction at neutral pH with intermediate B prepared by reacting compound C with excess methylamine (0.2 M) at pH 11.1.

Wide-band ^{1}H-decoupled ^{13}C NMR and ^{1}H NMR spectra of compound C were obtained with a Brucker WM-300 (300-MHz FT) spectrophotometer. All other ^{13}C NMR data were obtained with a Brucker HX-90 (90-MHz FT) instrument. Infrared spectra were recorded on a Beckman IR 4250 spectrophotometer.

Results and Discussion

The following results indicate that exposure of 3,10-dimethyl-5-deazaisoalloxazine 4a,5-epoxide to alkaline conditions initiates a remarkable ring contraction reaction to yield 4-methyloxazolo[4,5-*b*]quinolin-2(4*H*)-one (compound C, Scheme II).[7] The conversion of 3,10-dimethyl-5-deazaisoalloxazine 4a,5-epoxide to compound C is accompanied by the loss of one of the two methyl groups in the parent compound, as evidenced by ^{1}H NMR and ^{13}C NMR (Figure 1) data. The methyl group is lost from position N(3) since an identical product is formed with 10-methyl-5-deazaisoalloxazine 4a,5-epoxide. The even number obtained for the molecular weight of compound C (M^+ = 200) indicates that the compound must contain an even number of (or zero) nitrogen atoms. Intermediate B can be formed by reacting compound C with excess methylamine (Figure 2). Methylamine has also been identified as a product in the reaction with 3,10-dimethyl-5-deazaisoalloxazine 4a,5-epoxide. The results indicate that position N(3) in the epoxide is eliminated as RNH_2 (R = CH_3, H). That formation of compound C also involves loss of one of the two carbonyl groups in the epoxide is consistent with the following observations. Only 11 carbon atoms are detected

(6) Fersht, A. "Enzyme Structure and Mechanism"; W. H. Freeman: San Francisco, 1977; pp 163–164.

(7) Instability of the pyrimidine ring in certain flavin derivatives has been reported. For examples, see: Iwata, M.; Bruice, T. C.; Carrell, H. L.; Glusker, J. B. *J. Am. Chem. Soc.* **1980**, *102*, 5036–5044. Schonbrunn, A.; Abeles, R. H.; Walsh, C. T.; Ghisla, S.; Ogata, H.; Massey, V. *Biochemistry* **1976**, *15*, 1798–1807.

(8) Grande, H. J.; Gast, R.; van Schagen, C. G.; van Berkel, W. J. H.; Müller, F. *Helv. Chim. Acta* **1977**, *60*, 367–379.

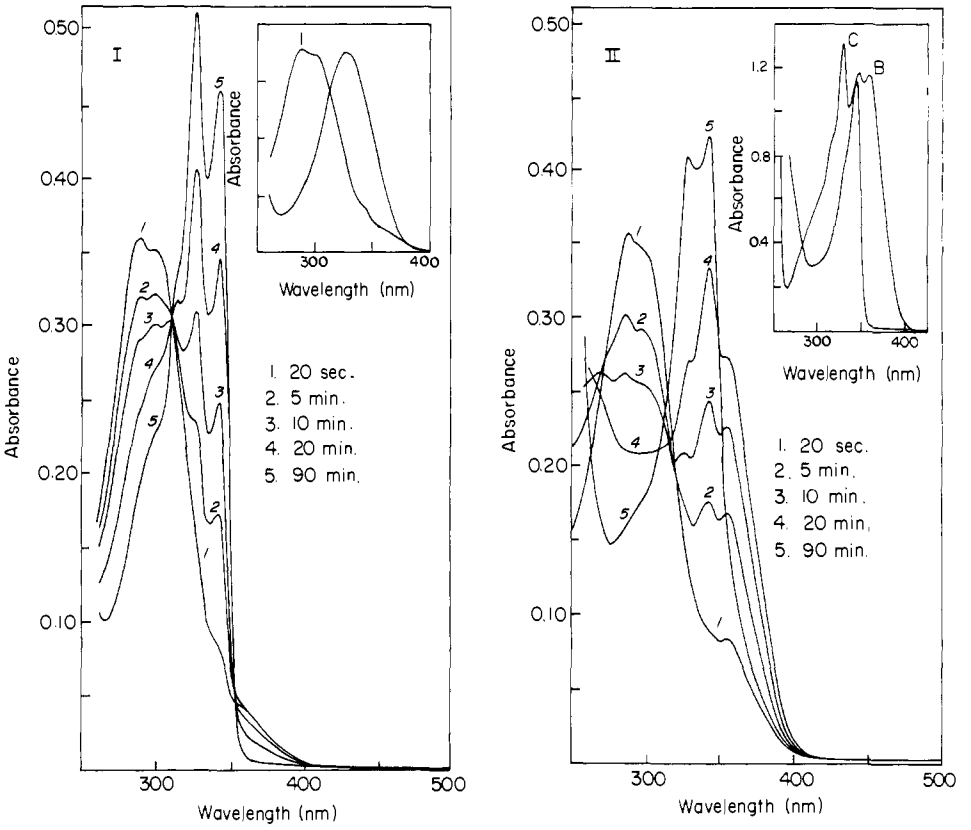


Figure 2. Reaction of 3,10-dimethyl-5-deazaisoalloxazine 4a,5-epoxide (22.6 μM) at pH 11.1 (I) or pH 12.2 (II). Conditions: 0.1 M sodium phosphate, 25 °C. (I) The spectrum of intermediate A (curve 1) is compared in the inset with the spectrum of the epoxide (22.6 μM) obtained at pH 7.0. Identical results are obtained under anaerobic conditions. (II) The reaction is not complete in 90 min, but the subsequent spectral course (not shown) is complicated by the instability of the product at pH 12.2. The inset shows the conversion of compound C (4.5×10^{-5} M) to intermediate B observed upon addition of methylamine (4.84 M) at pH 11.1.

in the ^{13}C NMR spectrum of compound C. The reaction is accompanied by the release of CO_2. Infrared absorption bands due to the two carbonyl groups in the parent compound (1650, 1712 cm^{-1}) are replaced by a "doublet" (1753, 1775 cm^{-1}) in compound C (Figure 1), similar to that observed for a single carbonyl group in related γ-lactones (e.g., 2(5*H*)-furanone: 1742, 1784 cm^{-1}), where the "doublet" is attributed to Fermi resonance.[9]

Compound C exhibits an intense purple fluorescence (emission λ_{max} = 368 nm) and an absorption spectrum (λ_{max} = 330, 345 nm ($\epsilon_{330} = 3.19 \times 10^4$ M^{-1} cm^{-1})) similar to that observed with deazaFMN·X at neutral pH (emission λ_{max} = 383 nm, absorption λ_{max} = 340, 356 nm ($\epsilon_{340} = 2.48 \times 10^4$ M^{-1} cm^{-1})).[1] Preliminary[10] and further studies in progress with deazaflavin·X strongly suggest tht it is analogous to compound C, differing only in the nature of substituents at positions 4, 6, and 7.

Immediate and quantitative conversion of the epoxide (λ_{max} = 331 nm) to intermediate A (λ_{max} = 290 nm) is observed at pH $\geq$11.1 (Figure 2, curve 1). Formation of intermediate A is not detectable at pH $\leq$9.1, where the epoxide slowly decays over a period of hours in a reaction accompanied by a loss of absorption at $\lambda > 300$ nm. At pH 10.1 intermediate A is detectable but the yield is lower than that observed at higher pH values owing to competing decomposition reactions. The spectral course of the reaction obtained with the epoxide at pH 11.1 (Figure 2) is similar to that observed at pH 2.8–9.2 for reactions initiated with intermediate A. This indicates that formation of intermediate A is the only step in the conversion of the epoxide to compound C that requires alkaline conditions.

Intermediate B is barely detectable at pH 11.1 and completely invisible at lower pH values owing to a rate-determining conversion of intermediate A to intermediate B. A plot of the observed rate constant for conversion of intermediate A to intermediate B (k_1) vs. pH yields a sigmoidal plot (Figure 3) which can be fitted by

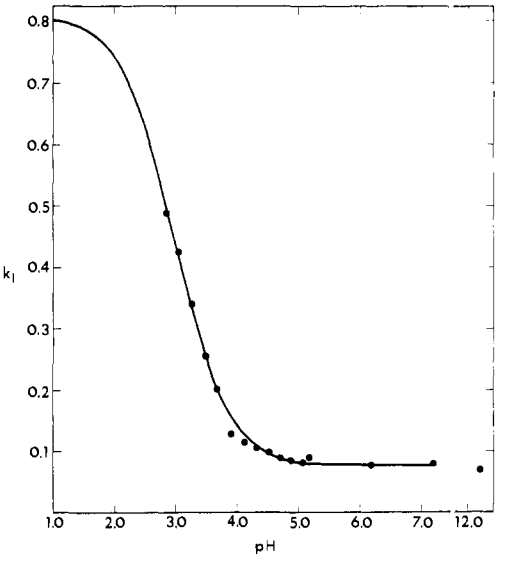
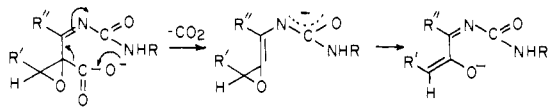


Figure 3. Effect of pH on the observed rate constant for conversion of intermediate A to intermediate B (k_1). The solid line is a theoretical curve obtained by a least-squares fit[11] of the data (pH 2.86–7.20) to a nonlinear function, $k_1 = (k_{1A} - K_A + k_{1HA}[H^+])(K_A + [H^+])^{-1}$. Reactions were monitored by using several concentrations of sodium citrate (37.5–150 mM, pH 2.86–5.08) or sodium pyrophosphate (37.5–112.5 mM, pH 5.18–7.20) buffer at constant ionic strength (μ = 1.06 with NaCl). A buffer dependence was observed, and the reported rate constants were obtained by extrapolation to zero buffer. (A buffer dependence was not observed at alkaline pH.) The value obtained for k_1 at pH 12.2 is included to show that the observed rate constant is essentially pH independent above neutral pH.

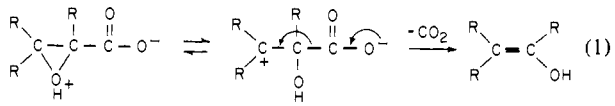
(9) Silverstein, R. M.; Bassler, G. C.; Morrill, T. C. "Spectrometric Identification of Organic Compounds"; Wiley: New York, 1974, pp 77–78, 149.

(10) Jorns, M. S.; Pokora, A.; Vargo, D. In "Flavins and Flavoproteins"; Massey, V., Williams, C., Eds.; Elsevier North Holland, 1982; pp 257–262.

Scheme III



a single acid dissociation constant (pK_a = 3.00, attributable to the carboxyl group) and rate constants for reaction with protonated (HA, k_{1HA} = 0.81 min^{-1}) and ionized (A^-, k_{1A^-} = 7.5 × 10^{-2} min^{-1}) forms of intermediate A. Although reaction of the neutral free acid is kinetically indistinguishable from a mechanism involving an oxirane-protonated glycidate salt, results[12] obtained for the decarboxylation of other simpler glycidic acid derivatives argue in favor of a zwitterionic mechanism (eq 1). The value obtained



(1)

for k_{1HA} is not very different from that observed for the decarboxylation of 2,3-epoxy-3-phenylbutanoic acid (k = 1.7 min^{-1}).[13] This suggests that the carbamoylimino group β to the carboxyl function in intermediate A has only a small effect on the reaction of the protonated intermediate. Although a slower rate is observed for the ionized intermediate, the latter exhibits enhanced reactivity as compared with the 2,3-epoxy-3-phenylbutanoate anion, where decarboxylation is not detectable.[13] This difference suggests that the β-carbamoylimino group may participate in the reaction with ionized intermediate A, possibly via a mechanism where decarboxylation precedes epoxide ring opening (Scheme III).

Intermediate B (λ_{max} = 346, 357 nm) is observed at pH >12 (Figure 2), where the rate of conversion of the intermediate to compound C (k_2) is slow and rate determining (k_1 = 6.7 × 10^{-2} min^{-1}, k_2 = 2.2 × 10^{-2} min^{-1}, pH 12.2).[14] Since similar values are observed for k_1 at pH 11.1 (6.6 × 10^{-2} min^{-1}) and pH 12.2, the change in the rate-determining step of the reaction in this pH range indicates that the rate of conversion of intermediate B to compound C increases with decreasing pH. This is consistent with the immediate conversion of intermediate B to compound C that is observed at neutral pH. Formation of intermediate B by reaction of compound C with excess methylamine is observed over a wider pH range (pH >10), and an intermediate with similar properties is detected at neutral pH in reactions observed with enzyme-bound epoxide, where the intermediate is subject to kinetic stabilization by the protein moiety. Studies to evaluate parameters affecting the stability of free vs. enzyme-bound intermediate B are in progress.[15]

Acknowledgment. This work was supported by a grant from the National Institutes of Health (GM22662). FT NMR spectra (300 MHz) were obtained at The Ohio State University Chemical Instrument Center (funded in part by National Science Foundation Grant CHE-7910019) with the help of Dr. C. E. Cottrel and Dr. A. G. Marshall.

Registry No. A, 82740-77-0; B, 82740-78-1; C, 80885-76-3; 3,10-dimethyl-5-deazaisoalloxazine 4a,5-epoxide, 72278-21-8; 10-methyl-5-deazaisoalloxazine 4a,5-epoxide, 72283-85-3; methylamine, 74-89-5; 3,10-dimethyl-5-deazaisoalloxazine, 38559-35-2.

(11) Bevington, P. R. "Data Reduction and Error Analysis for the Physical Sciences"; McGraw-Hill: New York, 1969; pp 237–240.
(12) Singh, S. P.; Kagan, J. *J. Org. Chem.* **1970**, *35*, 2203–2207.
(13) Shiner, V. J.; Martin, B. *J. Am. Chem. Soc.* **1962**, *84*, 4824–4827.
(14) (a) On the basis of the observed values for k_1 and k_2, a maximum yield for intermediate B at pH 12.2 can be calculated[14b] (58% at 25 min). The latter value corresponds to the time when absorbance at 375 nm reaches a maximum value. (b) Frost, A. A.; Pearson, R. B. "Kinetics and Mechanism"; Wiley: New York, 1961; p 168.
(15) The pyrimidine ring contraction reaction with 5-deazaflavin 4a,5-epoxide reported in this communication has been confirmed by Yoneda and Sakuma (Yoneda, F.; Sakuma, Y. *Tetrahedron Lett.* **1981**, *22*, 3977–3980) in a paper that appeared after this manuscript was submitted for publication. These workers proposed a different mechanism involving decarboxylation as a final step, which appears inconsistent with our studies, which show that the last step in the reaction is reversible in the presence of excess methylamine.

Resonance Raman Detection of an Fe–S Bond in Cytochrome $P450_{cam}$

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Abstract: Resonance Raman scattering experiments on isotopically labeled samples of the oxidized cytochrome $P450_{cam}$–substrate complex conclusively demonstrate the existence of an Fe–S bond that is sensitive to the presence of substrate. A general three-body oscillator model is developed that predicts the observed Raman frequency shifts of the 351-cm^{-1} Fe–S stretching mode due to the isotropic substitution.

We report results of resonance Raman scattering experiments on isotopically enriched (^{54}Fe, ^{34}S) and natural-abundance (^{56}Fe, ^{32}S) samples of the heme protein cytochrome $P450_{cam}$. These experiments provide the first direct and independent evidence of an Fe–S bond in the oxidized enzyme–substrate complex. The observed isotopic shifts of the 351-cm^{-1} Raman-active vibration are quantitated within a general three-body normal-mode oscillator model that is sensitive to the Fe–S–C bond angle. Moreover, the present data, along with previous work on the substrate-free enzyme, indicate that substrate binding produces a significant change in the iron–sulfur interaction.

Cytochrome P450 monoxygenases exhibit a close homology in catalytic and heme active-site parameters, despite a diverse range of xenobiotic detoxification, carcinogenic, and biosynthetic steroid functions.[1] Although the modes of electron transport and sub-

† Worcester Polytechnic Institute
‡ Cornell University.
§ University of Illinois.

(1) Coon, M. J.; White, R. E. In "Dioxygen Binding and Activation by Metal Centers"; Spiro, T. G., Ed.; Wiley: New York, 1980; p 73.

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