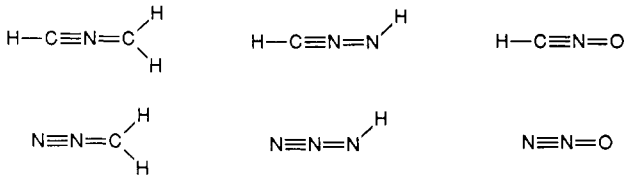
Table I footnotes (*Continued*)

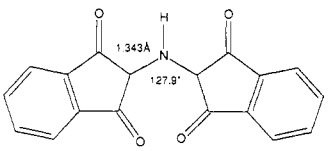
B: *Struct. Crystallogr. Cryst. Chem.* **1973**, *B29*, 237 and references therein. Inouye, Y. *Ibid.* **1984**, *C40*, 142. [i]Values for nitromethane from: Cox, A. P.; Waring, S. *Trans. Faraday Soc.* **1972**, *68*, 1060. NO bond length (1.202 Å) and ONO bond angle (130.6°) in $ClNO_2$ from: Miller, D. J.; Sinnot, K. M. *J. Chem. Soc.* **1958**, 350. Clayton, L.; Williams, Q.; Weatherly, T. L. *J. Chem. Phys.* **1959**, *30*, 1328; **1959**, *31*, 554. Oka, T.; Morino, Y. *J. Mol. Spectrosc.* **1963**, *11*, 349. NO bond length (1.180 Å) and ONO bond angle (136°) in FNO_2 from: Legon, A. C.; Millen, D. J. *J. Chem. Soc. A* **1968**, 1736. In $HONO_2$, 1.205 Å (average value) and 130.3°, from: Millen, D. J.; Morton, J. R. *J. Chem. Soc.* **1960**, 1523. Cox, A. P.; Riveros, J. M. *J. Chem. Phys.* **1965**, *42*, 3106. [j]Tranbarulo, R.; Ghosh, S. N.; Burrus, C. A., Jr.; Gordy, W. D. *J. Chem. Phys.* **1953**, *21*, 851. Hughes, R. H.; *Ibid.* **1953**, *21*, 959. Tanaka, T.; Morino, Y. *J. Mol. Spectrosc.* **1970**, *33*, 539.

electron 1,3-dipoles investigated appear to be properly described in terms of closed-shell (RHF) wave functions (Table I). On the other hand, open-shell (UHF) solutions are preferred for all 24-electron (allyl type) 1,3-dipoles considered.[9] Except for CH_2OO, energy differences for oxygen-centered dipoles are significantly larger than those for nitrogen-centered species, consistent with the notion that the more electronegative oxygen is less able than nitrogen to give up a π electron and bear positive charge, as demanded by closed-shell zwitterionic resonance structures.

Calculated heavy-atom bond lengths and skeletal bond angles for fulminic acid, diazomethane, hydrazoic acid, and nitrous acid (with 22 electrons) are in reasonable accord with their respective experimental structures. Comparison of calculated (and experimental) bond lengths for 1,3-dipoles with those found in two-heavy-atom hydrides[10] suggests that each of these molecules incorporates essentially a fully formed double bond *and* a fully formed triple bond. These molecules appear to be most appropriately described in terms of *hypervalent structures* in which the central nitrogen is allocated more than its normal complement of valence electrons.

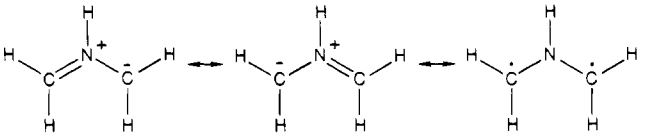


UHF/6-31G* geometries for 24-electron 1,3-dipoles are in reasonable accord with the limited experimental structural data. The calculated CN bond length (1.360 Å) and CNC bond angle (128.3°) for the parent azomethine ylide are quite close to those recently reported in the X-ray crystal structure of the substituted system.[11] While calculations for the parent nitrone yield CN and



NO bond lengths which fall slightly outside of the range of experimental distances for substituted systems, the UHF/6-31G* NO bond length and ONO bond angle in HNO_2 (1.219 Å and 125.8°, respectively) are in excellent agreement with values found in nitromethane (1.224 Å and 125.3°). The OO bond distance calculated for ozone at the UHF/6-31G* level (1.295 Å) is also in reasonable accord with the experimental value (1.272 Å), although the calculated bond angle (111.6°) is significantly smaller than the experimental quantity (116.8°).

Aside from the nitro compound (the NO bond lengths of which are typical of normal double linkages), which appears to be most appropriately described as a hypervalent molecule, the calculated (and experimental) equilibrium structures for all of the 24-electron 1,3-dipoles incorporate two bonds which are midway between normal single and double linkages. This suggests description of this species, and of the other 22-electron 1,3dipoles, in terms of resonance structures in which the central atom is normal valent.



Structural differences between 22- and 24-electron dipoles may be rationalized in terms of differences in bond energies.[12] Hypervalent descriptions for 22-electron 1,3-dipoles each incorporate either a strong CN or a strong NN triple bond, whereas expansion of the valence octet about the central element in the 24-electron species results at best in the creation of two double bonds.

Acknowledgment. This research was supported in part by grants from the National Science Foundation. S.D.K. thanks the Control Data Corporation for a PACER fellowship.

(8) Two or more geometrical (cis–trans) isomers exist for some of the systems investigated. Only the lowest energy structure (at UHF/6-31G) has been considered and has been depicted in Table I. A detailed description of the energy surfaces for 1,3-dipolar species will appear in our upcoming full paper.

(9) These same trends have also been uncovered by Hiberty and Leforestier [Hiberty, P. C.; Leforestier, C. *J. Am. Chem. Soc.* **1978**, *100*, 2012] who performed RHF/STO-3G calculations followed by 6 × 6 CI on a selection of 1,3-dipolar intermediates. Specific diradical and zwitterionic contributions were assessed by expansion in terms of valence-bond wave functions. Hiberty and Leforestier noted that the diradical contributions to the 24-electron 1,3-dipoles ranged between 43% and 59% of the total (the largest contribution being found for ozone), while the corresponding contributions for 22-electron 1,3-dipoles ranged from 17% to 32%.

(10) For a summary, see: Hehre, W. J.; Radom, L.; Schleyer, P. v. R.; Pople, J. A. *Ab Initio Molecular Orbital Theory*; Wiley: New York, 1986; p 146ff.

(11) Grigg, R.; Maloney, J. F.; Mongkolaussavaratana, T.; Thianpatanagul, S. *J. Chem. Soc., Chem. Commun.* **1986**, 421.

(12) Dobbs, K. D.; Kahn, S. D.; Hehre W. J.; Pople, J. A., unpublished results.

Stereochemical Course of the Cryptic Elimination and Cyclization Steps in the Reaction Catalyzed by Dehydroquinate Synthase

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In a pivotal transformation in the shikimate pathway,[1] dehydroquinate synthase catalyzes the conversion of the seven-carbon keto acid 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate (DAHP, **1**) to dehydroquinate (DHQ, **2**), the first carbocyclic metabolite in the biosynthesis of the three aromatic amino acids. Previous studies suggested that the enzymatic reaction may proceed in five steps. First, oxidation of the hydroxyl group at C-5 by a bound NAD^+ would facilitate the β-elimination of phosphate; reduction at C-5, followed by ring opening, would then lead to an internal aldol reaction producing DHQ (Scheme I).[2] While this proposal accommodates the limited mechanistic information available,[2,3] none of the postulated intermediates have

(1) (a) Haslam, E. *The Shikimate Pathway*; Wiley: New York, 1974. (b) Weiss, U.; Edwards, J. M. *The Biosynthesis of Aromatic Compounds*; Wiley: New York, 1980.

(2) (a) Srinivasan, P. R.; Rothschild, J.; Sprinson, D. B. *J. Biol. Chem.* **1963**, *238*, 3176. (b) Rotenberg, S. L.; Sprinson, D. B. *Proc. Natl. Acad. Sci. U. S. A.* **1970**, *67*, 1669.

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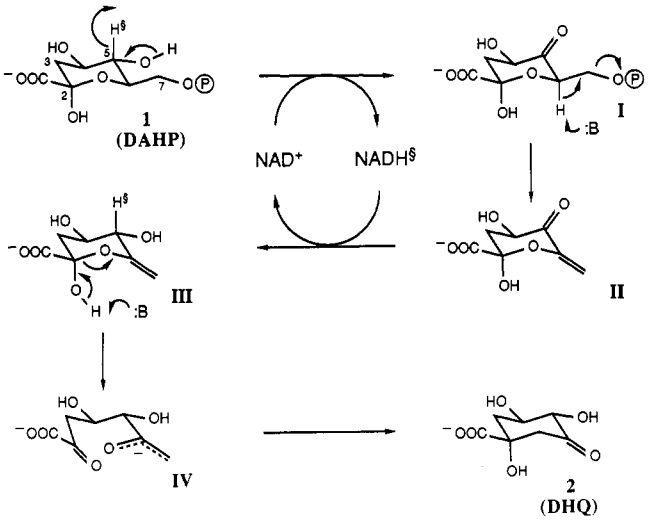
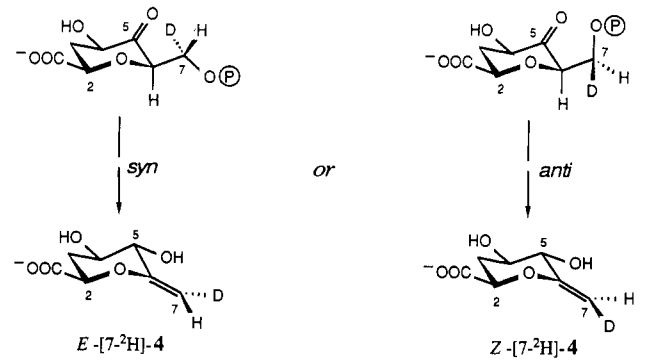
Scheme I

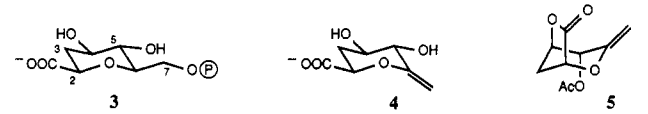


Scheme II

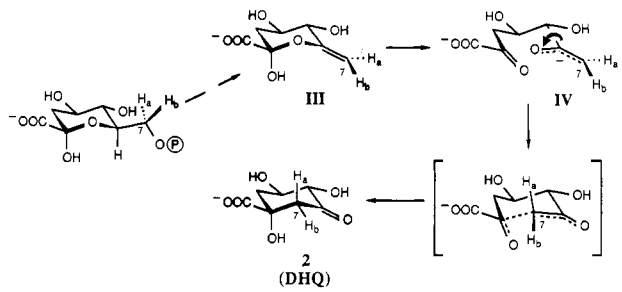


been isolated, and additional data are needed to allow a proper description of this unusually complex sequence. We report here the use of the 2-deoxy substrate analogue **3** to address three questions. First, when in the reaction sequence does ring opening occur?[3d] Second, what is the stereochemical course of the postulated β-elimination? Third, given that inversion at C-7 occurs during the overall conversion of DAHP to DHQ,[2b] what is the transition-state geometry of the aldol reaction?

Incubation of **3**[4] with DHQ synthase[6] results in the formation of inorganic phosphate (at about 2% of the rate observed for the natural substrate DAHP[7]) and of the enol ether **4**.[8] DHQ synthase evidently catalyzes the elimination of phosphate from the analogue **3**, presumably by effecting the first three steps of the normal pathway (oxidation, elimination, and reduction; Scheme I). The facility with which this occurs with a molecule (**3**) that is constrained to be cyclic suggests that when DAHP is the substrate, ring opening does not occur until after the reduction step, as illustrated in Scheme I.



Scheme III



On the reasonable basis that **4** is produced by the same sequence of enzymic steps that produce the intermediate III, we can use stereospecifically monodeuteriated [7-^{2}H]-**3** to determine the stereochemical course of the elimination step (Scheme II). The analysis is straightforward provided that the (*E*)- and (*Z*)-vinyl protons can be unambiguously assigned in the ^{1}H NMR spectrum of **4**. Unfortunately, these two resonances are only 0.03 ppm apart and also show equal long-range couplings to the allylic proton on C-5. To assign the vinyl proton resonances, therefore, **4** was converted to the bicyclic lactone **5**,[9] in which the *E*,*Z* assignment was readily accomplished by NOE difference spectroscopy.[10]

The synthesis of (7*S*)-[7-^{2}H]-**3** proceeded from β-D-(6*S*)-[6-^{2}H]glucose pentaacetate[11] via (6*S*)-[6-^{2}H]-2-deoxyglucose tetraacetate.[12] Further transformation as for the unlabeled molecule[4] provided (7*S*)-[7-^{2}H]-2-deoxy-DAHP. Incubation of this material with DHQ synthase provided [7-^{2}H]-**4**, which was isolated and converted to [7-^{2}H]-**5**. NMR analysis indicated that the deuterium label was entirely in the *E* position, which establishes that *DHQ synthase catalyzes the syn elimination of inorganic phosphate.*

Since the *overall* reaction inverts the configuration at C-7 (Scheme III),[2b] knowledge of the geometry of phosphate elimination limits the possible transition states for the subsequent aldol cyclization. There are only three transition states that satisfy the stereochemical requirements: two of boatlike geometry and one of chairlike geometry. Consideration of steric effects and of minimal motion strongly favors the chairlike conformation illustrated in Scheme III, which can be reached by a rotation of 180° about only one bond (between C-5 and C-6).[13]

Uncovering the stereochemical secrets of the DHQ synthase reaction therefore reveals a β-elimination that is syn and an aldol transition state that is chairlike. These results are in gratifying accord with enzymic precedent and chemical expectation. Thus, enzyme-catalyzed β-dehydrations where the abstracted proton (as

(3) (a) Le Maréchal, P.; Azerad, R. *Biochimie* **1976**, *58*, 1123. (b) Rotenberg, S. L.; Sprinson, D. B. *J. Biol. Chem.* **1978**, *253*, 2210. (c) Maitra, U. S.; Sprinson, D. B. *J. Biol. Chem.* **1978**, *253*, 5426. (d) Lambert, J. M.; Boocock, M. R.; Coggins, J. R. *Biochem. J.* **1985**, *226*, 817.

(4) Treatment of 2-deoxyglucose tetraacetate with Me_3SiCN and BF_3·Et_2O in nitromethane afforded the epimeric 1-nitrilo-1,2-dideoxyglucose triacetates.[5] Hydrolysis of this mixture (1 M KOH, MeOH–THF) to the carboxylic acid, followed by esterification (CH_2N_2), gave the carboxymethyl ester, which was purified as its triacetate. Phosphorylation of the primary hydroxyl group by diphenylphosphochloridate and reacetylation provided the carboxymethyl phosphate triester diacetate in 36% yield. Hydrogenolysis (in MeOH, over PtO_2: Van Boom, H. H.; deRooy, J. F. M.; Reese, C. B. *J. Chem. Soc., Perkin Trans. 1* **1973**, 2513) and saponification then provided the target compound **3**. Satisfactory spectral data were obtained for all new compounds.

(5) Grynkiewicz, G.; BeMiller, J. N. *Carbohydr. Res.* **1982**, *108*, 229.

(6) Using homogeneous enzyme (specific activity 40 units/mg), obtained from our overproducing strain *E. coli* RB 791 (pJB14): Frost, J.; Bender, J. L.; Kadonaga, J. T.; Knowles, J. R. *Biochemistry* **1984**, *23*, 4470.

(7) The rate of P_i release was followed at saturating substrate levels, 15 °C, in 0.10 M 3-(*N*-morpholino)propanesulfonate (MOPS) buffer, pH 7.4, containing NAD^+ (0.15 mM) and Co^{2+} (1.0 mM). Under these conditions, the K_m for **3** is 100 μm, while the K_m for **1** is <10 μM (the lower limit of the analytical method: Ames, B. N. *Methods Enzymol.* **1966**, *8*, 115).

(8) ^{1}H NMR for **4** (D_2O, 500 MHz, referenced to HOD at 4.68 ppm): δ 4.60 (t, 1 H, *J* = 1.7 Hz, C_7–H), 4.57 (t, 1 H, *J* = 1.7 Hz, C_7–H), 3.87 (dd, 1 H, *J* = 12.3 and 2.4 Hz, C_2–H), 3.76 (dt, 1 H, *J* = 9.5 and 1.7 Hz, C_5–H), 3.53–3.58 (m, 1 H, C_4–H), 2.26 (ddd, 1 H, *J* = 13.1, 4.9, and 2.4 Hz, equatorial C_3–H), 1.60 (q, 1 H, *J* = 12.2 Hz, axial C_3–H).

(9) Treatment of the tetramethylammonium salt of the enol ether **4** with excess acetic anhydride in pyridine yielded the lactone **5** as the sole organic-soluble product. ^{1}H NMR for **5** ($CDCl_3$, 500 MHz) δ 5.57 (dd, 1 H, *J* = 4.3 and 1.4 Hz, C_5–H), 4.90 (d, 1 H, *J* = 1.8 Hz, C_7–H), 4.87 (br t, 1 H, *J* = 5 Hz, C_4–H), 4.72 (d, 1 H, *J* = 1.8 Hz, C_7–H), 4.48 (br d, 1 H, *J* = 3.3 Hz, C_2–H), 2.71 (dd, 1 H, *J* = 13.3 and 0.9 Hz, axial C_3–H), 2.23 (dddd, 1 H, *J* = 13.3, 5.5, 3.3, and 1.5 Hz, equatorial C_3–H), 2.12 (s, 3 H, CH_3).

(10) Irradiation of the C_5–H resonance at 5.57 ppm resulted in ~3% enhancement of the doublet at 4.72 ppm, allowing the assignment of this resonance to the (*E*)-vinyl proton.

(11) Ohrui, H.; Horiki, H.; Kishi, H.; Meguro, H. *Agric. Biol. Chem.* **1983**, *47*, 1101.

(12) (a) Roth, W.; Pigman, W. *Methods Carbohydr. Chem.* **1963**, *2*, 405. (b) Overend, W. G.; Stacey, M.; Staněk, J. *J. Chem. Soc.* **1949**, 2841.

(13) Had the elimination of phosphate proceeded anti, the aldol transition state predicted by minimal motion would have been boatlike.

here) is α to a ketone or a thiol ester are invariably syn,[14] whereas those where the abstracted proton is α to a carboxylic acid are universally anti.[14] Further, a chairlike aldol transition state is most reasonable in the light of the preferred chair conformation of the final product, dehydroquinate.[15]

Acknowledgment. This work was supported by the National Institutes of Health.

(14) For a recent list. see: Schwab, J. M.; Klassen, J. B.; Habib, A. *J. Chem. Soc., Chem. Commun.* **1986**, 357.
(15) Haslam, E.; Turner, M. J.; Sargent, D.; Thompson, R. S. *J. Chem. Soc. C* **1971**, 1489.

A New Semisynthetic Esterase

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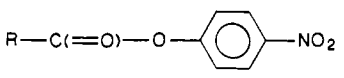
In recent years there has been considerable effort to mimic the catalytic action of enzymes. Most attempts have concentrated on ester hydrolases. Binding cavities based on macrocyclic structures[1,2] provide high rate accelerations for ester hydrolysis; however, these catalysts remain permanently acylated and no turnover is possible. Poly(ethylenimines) with attached imidazole groups[3] and micellar systems[4] have been shown to catalyze ester hydrolysis with turnover for some substrates, but the rate enhancement and the binding capability are only moderate. In the present paper we describe a new semisynthetic esterase that (1) exhibits some of the highest acylation rate enhancements observed with synthetic catalysts, (2) supplies an excellent binding site with binding free energies similar to those of enzymes, and (3) provides fast overall turnover.

The semisynthetic enzyme comprises a nonenzymatic protein, sperm-whale Met-myoglobin, from which the heme group has been removed by the acid/acetone method.[5] The apoprotein thus obtained possesses a deep hydrophobic cavity which serves as an enzymatic binding site for hydrophobic substrates. Within the heme pocket of myoglobin there are two imidazole residues, an established ester hydrolyzing catalyst. As expected, this combination of cavity and catalytic groups exhibits excellent hydrolyzing capability for esters with the structure **1**.



1

1a, R = C_5H_7, PNPcaproate
1b, R = C_9H_{11}, PNPcaprate
1c, R = CH_3, PNPacetate

The rates of hydrolysis in the presence of either apo-Mb or free imidazole (0.05 M Tris, pH 8.0, at 25 °C) measured by following the formation of *p*-nitrophenolate ion (PNP) at 400 nm were corrected for spontaneous hydrolysis in the buffer. PNPcaproate (**1a**) (1×10^{-5} M) hydrolyzes in the buffer with a first-order rate constant of 2.6×10^{-5} s^{-1}. In the presence of the apoprotein the hydrolysis is greatly accelerated and exhibits saturation behavior. The data obtained with 2×10^{-5} to 11×10^{-5} M of apo-Mb fit a Lineweaver–Burk plot, thus demonstrating 1:1 complex formation. These results are consistent with the first two steps in a mechanism analogous to that of hydrolytic enzymes (Scheme I). The value of k_2 for **1a** (4.9×10^{-2} s^{-1}) is 1900 times faster

Scheme I

$$\text{Apo-Mb} + \text{PNPAc} \overset{K_s}{\rightleftharpoons} \text{Apo-Mb-PNPAc}$$

$$\text{Apo-Mb-PNPAc} \xrightarrow{k_2} \text{Apo-Mb-Ac} + \text{PNP}$$

$$\text{Apo-Mb-Ac} \xrightarrow{k_3} \text{Apo-Mb} + \text{acid}$$

than k_2 in the buffer, and the Michaelis constant, K_m, is 7.4×10^{-5} M. The "catalytic constant", $k_2/K_m = 660$ M^{-1} s^{-1}, is 3000 times larger than $k_{imidazole}$ (0.22 M^{-1} s^{-1}) and 15 times larger than the one observed in the hydrolysis of the same substrate by the PEI–imidazole "synzyme".[3] The more hydrophobic substrate **1b**, studied under identical conditions, manifested the same kinetic behavior. Similar k_2 and K_m values were observed, 0.030 s^{-1} and 6.1×10^{-5} M, respectively; however, the enhancements achieved for this ester, $k_2/k_{buffer} = 4900$ and $(k_2/K_m)/k_{imidazole} = 3780$, are even higher than with **1a**.

The hydrolysis of PNPacetate (**1c**) by the apo-Mb semisynthetic enzyme was studied with ester concentrations in large excess over that of the enzyme. The initial rates of PNP release obtained with 3.6×10^{-5} M apo-Mb and 2.0×10^{-4} to 13.0×10^{-4} M **1c** fit a Lineweaver–Burk plot and yielded $k_2 = 5.8 \times 10^{-3}$ s^{-1} and $K_m = 4.3 \times 10^{-4}$ M. The rate enhancement ($k_2/k_{buffer} = 42$ or $(k_2/K_m)/k_{imidazole} = 27$) achieved by the semisynthetic enzyme for hydrolysis of **1c** is only moderate; however, it is almost 5 times larger than the one obtained with β-cyclodextrin.[6,7] From comparison of the "catalytic constants", k_2/K_m, under similar conditions, the apo-Mb is 287 times more efficient than cyclodextrin in hydrolyzing PNPacetate. Moreover, while PNPacetate permanently acetylates cyclodextrin, thus disabling the catalyst, the apo-Mb hydrolysis of PNPacetate is fully catalytic with a deacylation rate faster than acylation. Turnover was demonstrated by comparing the rate of hydrolysis of 1×10^{-5} M **1c** by 5×10^{-5} M apo-Mb to that of 1×10^{-5} M **1c** by a reaction mixture of 5×10^{-5} M apo-Mb and 5×10^{-5} M **1c** which had been allowed to react to 95% completion. The rates were identical, verifying that acylation rather than deacylation is the rate-determining step, i.e., $k_3 \gg k_2$. In the case of **1a** and **1b**, the same type of experiment revealed a decrease in catalysis rate, suggesting that with these two esters the deacylation is rate limiting. Preliminary studies of the absorbance changes at 240–270 nm, the acylated imidazole peak, indicate that the acyl group is not permanently attached and hydrolyzes slowly.

It is well established that peripheral nucleophilic residues of proteins like histidine, lysine, or serine can catalyze PNP release from PNP esters.[8] In fact, met-Mb in its native and denatured forms has been shown to react with **1c** but with a very slow rate.[9] In order to ascertain that our remarkable catalytic rates result from one active site only, namely, the empty heme pocket, we reinvestigated those myoglobin ester interactions under our experimental conditions. When **1a** was hydrolyzed in the presence of either native Mb or 8 M urea-denatured Mb, only a marginal increase in rate compared to the buffer was observed. In both cases the rate of hydrolysis was more than 3 orders of magnitude slower than in the presence of apo-Mb. This result establishes the heme cavity as the active site of the semisynthetic esterase. The existence of turnover supports the claim that one or both of the imidazole groups in the pocket are responsible for the hydrolase activity.

The apoprotein ester dissociation constants of (6.1×10^{-5})–(4.3×10^{-4}) M are significantly lower than those observed with cy-

(1) Cram, D. J.; Katz, H. E. *J. Am. Chem. Soc.* **1983**, *105*, 135–137.
(2) Trainor, G. L.; Breslow, R. *J. Am. Chem. Soc.* **1981**, *103*, 154–158.
(3) Klotz, I. M.; Royer, G. P.; Scrapa, J. S. *Proc. Natl. Acad. Sci. U.S.A.* **1971**, *68*, 263–264.
(4) Menger, F. M.; Whitesell, L. G. *J. Am. Chem. Soc.* **1985**, *107*, 707–708.
(5) Rossi-Fanelli, A.; Antonini, E.; Caputo, A. *Biochim. Biophys. Acta* **1958**, *301*, 608.
(6) Taniguchi, Y.; Makimoto, S.; Suzuki, K. *J. Phys. Chem.* **1981**, *85*, 3469–3472.
(7) Bender, M. L.; Turnquest, B. W. *J. Am. Chem. Soc.* **1957**, *79*, 1652–1655.
(8) For example: (a) Bruice, T. C.; Holmquist, B.; Stein, P. *J. Am. Chem. Soc.* **1967**, *89*, 4221–4222. (b) Piszkiewicz, D.; Bruice, T. C. *Biochemistry* **1968**, *7*, 3037–3047. (c) Means, G. E.; Bender, M. L. *Biochemistry* **1975**, *14*, 4989–4994.
(9) Breslow, E.; Gurd, F. R. *J. Biol. Chem.* **1962**, *237*, 371–381.

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