$$CH_3O\cdot + 1e^- \rightarrow CH_3O^- \quad (5)$$

The easy photoreduction that we observed for O=Mo^V(TPP)—OCH_3 may account for the spectral change caused by laser irradiation of O=Mo^V(OEP)—OCH_3 reported by Ohta et al.[21] during the measurement of resonance Raman spectra in KBr pellets. The appearance of a new band at 956 cm^{-1} is in close agreement with the value of the Mo—O stretch observed in infrared spectroscopy at 965 cm^{-1} for O—Mo^{IV}(OEP).[22]

This also gives a reasonable pathway for the photodecomposition of the diperoxomolybdenum(VI) porphyrin $(O_2)_2Mo^{VI}$(TPP). We can assume, as favored by extended Hückel calculations,[23] that the first step is the homolysis of a Mo–O bond as shown in Scheme I, affording formally a peroxo-superoxomolybdenum(V) complex. This intermediate should be very unstable as there is no example of a fully characterized peroxo complex of d^n, n odd, transition metal[24] and recently an oxomolybdenum(V) porphyrin complex has been reported to be reduced by superoxide ion.[25] Next step will be the departure of molecular oxygen providing a peroxomolybdenum(IV) complex which will give the *cis*-dioxomolybdenum(VI) porphyrin after a very thermodynamically favorable internal redox reaction.[23]

These results clearly establish the ability of molybdenum porphyrins to harvest solar energy to produce reactive intermediates by redox processes.

Acknowledgment. This research was supported by the Centre National de la Recherche Scientifique. We thank Dr. I. Tkatchenko for helpful discussions and Dr. R. Hoffmann for communicating us his results concerning the theoretical calculations on molybdenum porphyrins. We are indebted to Dr. J.-P. Puaux for his expert assistance in quantum measurements which have been performed at the Laboratoire de Cinétique et de Génie Chimique de l'I.N.S.A. de Lyon.

(21) Ohta, N.; Scheurmann, W.; Nakamoto, K.; Matsuda, Y.; Yamada, S.; Murakami, Y. *Inorg. Chem.* **1979**, *18*, 457–460.
(22) Buchler, J. W.; Eikelmann, G.; Puppe, L.; Rohbock, K.; Schneehage, H. H.; Weck, D. *Liebigs Ann. Chem.* **1971**, *745*, 135–151.
(23) Tatsumi, K.; Hoffmann, R. Private communication.
(24) Hoffmann, R.; Chen, M. M.-L.; Thorn, D. L. *Inorg. Chem.* **1977**, *16*, 503–511.
(25) Imamura, T.; Numatatsu, T.; Terui, M.; Fujimoto, M. *Bull Chem. Soc. Jpn.* **1981**, *54*, 170–174.

Semisynthetic Enzymes: Synthesis of a New Flavopapain with High Catalytic Efficiency

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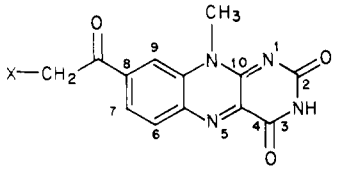
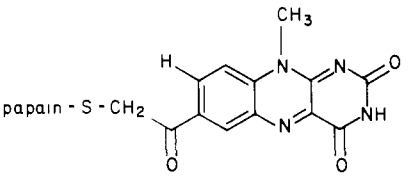
Flavopapain **1**, derived from alkylation of the active site cysteine-25 of papain [EC 3.4.4.10] with the reactive flavin analogue **2**, is a highly efficient semisynthetic enzyme, whose catalytic activity surpasses that of our best previously described flavopapain **4**[1-3] by over an order of magnitude and begins to approach the activity exhibited by all but the most efficient flavin-containing oxidoreductases. We feel that this represents a significant step toward the production of enzymelike molecules by a process that we call "chemical mutation". This is an approach to the design of catalysts which relies on the combination of an existing protein-binding site with a chemically reactive coenzyme analogue,



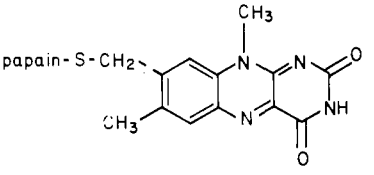
1, X = papain-S-
2, X = Br
3, X = H



4

thus exploiting the binding specificity of the protein but expressing the characteristic chemical reactivity of the covalently attached coenzyme.

It has been demonstrated that flavopapain **4** is an effective catalyst for the oxidation of N^1-alkyl-1,4-dihydronicotinamides by O_2.[1-3] Rates of the enzymatic oxidation exceed those of the flavin prosthetic group by an order of magnitude. More importantly, catalysis by the semisynthetic enzyme **4** exhibited substrate saturation kinetics, as would be expected for an enzymatic reaction involving the obligatory participation of a binding site. In contrast, oxidation reactions catalyzed by flavopapain **5** exhibited modest rate enhancements of the order of about 3-fold and did not exhibit saturation kinetics.



5

The difference between flavopapains **4** and **5** had been anticipated on the basis of model building, assuming that the carbonyl oxygen of **4** could hydrogen bond to the peptide backbone of papain, thus positioning the flavin within the hydrophobic binding groove of the enzyme.[4] No such interaction is possible in the case of **5**, and hence no comparable positioning of the flavin prosthetic group seems likely in this case.

Further model building, this time using structurally similar 8-acetyl-10-methylisoalloxazine attached as in **1**, with the acetyl carbonyl constrained to participate in a scheme of hydrogen bonding identical with that postulated to occur in the case of **4**, led to the prediction that N^1-benzyl-1,4-dihydronicotinamide (N^1-BzNH) could fit snugly within the binding pocket of the enzyme, in close proximity to the flavin. Hence, the prediction was made that flavopapain **1** should be a good catalyst for the oxidation of this substrate and fall into that group of semisynthetic enzymes which exhibits saturation kinetics and relatively high catalytic rate enhancements.

In order to test this prediction, a synthesis of the requisite 8-acetyl-10-methylisoalloxazine **3** had to be devised. This was achieved starting with the well-characterized-3-amino-4-nitroacetophenone **6**[5] as shown in Scheme I.

Treatment of amine **6** with trifluoroacetic anhydride in a solvent of trifluoroacetic acid at 0 °C for 20 min led to the isolation of 3-(trifluoroacetamido)-4-nitroacetophenone as short yellow needles

(1) Levine, H. L.; Nakagawa, Y.; Kaiser, E. T. *Biochem. Biophys. Res. Commun.* **1977**, *76*, 64–69.
(2) Levine, H. L.; Kaiser, E. T. *J. Am. Chem. Soc.* **1978**, *100*, 7670–7677.
(3) Kaiser, E. T.; Levine, H. L.; Otsuki, T.; Fried, H. E.; Dupeyre, R. *Adv. Chem. Ser.* **1980**, *191*, 35–48.

(4) Papain derivatives which result from the alkylation of Cys-25 with chloromethyl ketone substrate analogues have been examined structurally via X-ray diffraction. It was found that the carbonyl oxygen derived from the chloromethyl ketone was within hydrogen-bonding distance of two N–H groups: the side-chain NH of Gln-19 and the backbone NH of Cys-25. Drenth, J.; Kalk, K. H.; Swen, H. M. *Biochemistry* **1976**, *15*, 3731–3738.
(5) Waters, W. A. *J. Chem. Soc.* **1945**, 629.

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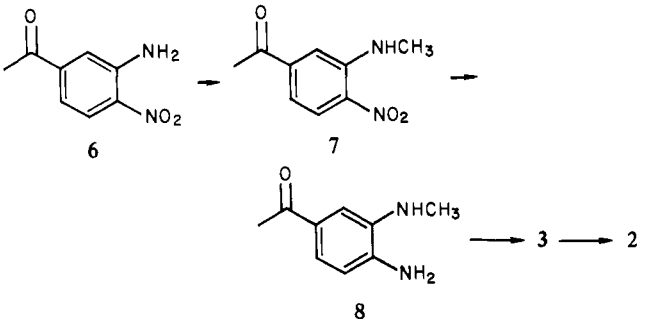
Table I. Rate Parameters for the Oxidation of Dihydronicotinamides by Flavopapain 1 and the Model Flavin 8-Acetyl-10-methylisoalloxazine 3 at 25 °C and pH 7.5[a]

dihydronicotinamide	enzymatic reaction K_m, μM	enzymatic reaction k_{cat}, s^{-1}	enzymatic reaction k_{cat}/K_m, M^{-1} s^{-1}	model reaction second-order rate constant k, M^{-1} s^{-1}	enzymatic rate enhancement
N^1-BzNH (9)	2.7 ± 0.3	0.093 ± 0.009	33 800	170 ± 2	199
by initial rates	2.7 ± 0.5	0.090 ± 0.007	32 900		
N^1-PrNH (10)	0.81 ± 0.08	0.048 ± 0.003	58 700	878 ± 23	66
by initial rates	0.77 ± 0.095	0.047 ± 0.0008	61 300		
N^1-HxNH (11)[b]	0.12 ± 0.01	0.067 ± 0.009	570 000	917 ± 18	621
NADH[c]	340 ± 30	0.0073 ± 0.0005	21	5.12	4

[a] Measured in 0.1 M Tris·HCl, 0.1 mM EDTA containing 0–1% ethanol. Model reaction also contains 0–1% Me_2SO. Each solution of 3.0 mL contains 0.01 mg (10 units) of superoxide dismutase and 0.1 mg (3500 units) of catalase. [b] Measured in a 5-cm-path-length cell at an ambient temperature of 27 °C. Other conditions as in footnote *a*. [c] Measured in the absence of superoxide dismutase and catalase.

Scheme I



in 92% yield: mp 107 °C; NMR ($CDCl_3$) δ 2.65 (3 H, s), 7.83 (1 H, dd, J = 8.75 and 2 Hz), 8.33 (1 H, d, J = 8.75 Hz), 9.18 (1 H, d, J = 2 Hz), 11.17 (1 H, br). This amide was methylated by treatment with methyl iodide and powdered KOH in refluxing acetone for 30 min.[6] The protecting group was removed by passing the resultant crude product through a column of alumina to give 3-(methylamino)-4-nitroacetophenone **7**, isolated in 75% yield as red crystals: mp 110.5–111 °C;[7] NMR ($CDCl_3$) δ 2.61 (3 H, s), 3.08 (3 H, d, J = 5 Hz), 7.13 (1 H, dd, J = 2 and 9 Hz), 7.4 (1 H, d, J = 2 Hz), 7.93 (1 H, br), 8.21 (1 H, d, J = 9 Hz). A solution of **7** in ethanol was next treated with hydrogen gas using a catalyst of 10% Pd on C at atmospheric pressure and room temperature for 4 h. The resultant diamine **8** was not isolated, but was immediately added to a stirred mixture of finely powdered alloxan monohydrate in concentrated HCl, brought to a boil, and refluxed for 20 min. After cooling to 0 °C, product was isolated by filtration. The 8-acetyl-10-methylisoalloxazine **3** was thus obtained as a fine yellow powder[7] in 51% yield: NMR (trifluoroacetic acid) δ 3.11 (3 H, s), 4.83 (3 H, s), 9.0 (2 H, m), 9.28 (1 H, m); electronic absorption spectra λ_{max} (ε, M^{-1} cm^{-1}) (50 mM phosphate, pH 7.0; 1% Me_2SO) 442 nm (10 300), 341 (9 450), 244 (26 100). The acetylflavin **3** was monobrominated by treating a solution of **3** in acetic acid at 90 °C with 1.1 equiv of bromine. The (bromoacetyl)flavin **2** precipitated from solution and was isolated by filtration as the HBr salt in 67% yield: NMR (trifluoroacetic acid) δ 4.91 (3 H, s), 4.97 (2 H, s), 9.11 (2 H, m), 9.41 (1 H, m).

For modification, freshly purified papain in deionized water[8] (at a concentration of approximately 2×10^{-5} M) was treated with a fivefold molar excess of (bromoacetyl)flavin **2**. Reaction was allowed to proceed for 3–5 h or until no further decrease in enzymatic activity was detected[9] and quenched using an amount of cysteine equimolar to the amount of bromoflavin. Modification was repeated once or twice more, until the activity of the papain was reduced to less than 5% of the original. Untreated flavin was removed by dialysis against deionized water (2-L portions, changed 5 times over 3 days).

Gel filtration of the resultant flavopapain under denaturing conditions (Sephadex G-25, 0.2% sodium dodecyl sulfate) revealed that the remaining flavin chromophore eluted with the high molecular weight fraction. Further, sulfhydryl determination using Ellman's reagent before and after modification indicated a loss of 90% of the reactive sulfhydryl group. We interpret these measurements as indicating a covalent attachement of flavin via the Cys-25 as shown in structure **1**.

The electronic absorption spectra of flavopapain **1** appeared simply to correspond to the combination of the spectra of papain and that of flavin **3**, showing maxima at 276, 339 and 440 nm. Using the known extinction coefficient for papain and **3**, the molar ratio of flavin to papain could be determined. This varied from preparation to preparation but was always between 0.95 and 1.05.

Flavopapain **1** was found to catalyze the oxidation of N^1-alkyl-1,4-dihydronicotinamides[10] **9–11** as well as NADH. Under aerobic conditions with substrate in excess, the kinetics are readily explained by the rate expression 1, which is derived from eq 2a,b with $k_0[O_2] >> k_{cat}$. The kinetic constants were determined by

$$v = \frac{k_{cat}[E_0][N^1\text{-RNH}]}{K_m + [N^1\text{-RNH}]} \quad (1)$$

$$E\ (ox) + N^1\text{-RNH} \underset{K_m}{\rightleftharpoons} ES \xrightarrow{k_{cat}} EH_2\ (red) + N^1\text{-RN} \quad (2a)$$

$$EH_2\ (red) + O_2 \xrightarrow{k_0} E\ (ox) \quad (2b)$$

fitting the total time course of the reaction to eq 1 using an iterative curve-fitting computer program.[11] Results are presented in Table I. In the case of substrates **9** and **10**, kinetic constants were also determined by the method of initial rates.[12] Values derived from this analysis agreed well with those derived from analysis of the total time course.[13]

Oxidation of N^1-alkyldihydronicotinamides by model flavin **3** under aerobic conditions with dihydronicotinamide in excess proceeded with first-order kinetics. The pseudo-first-order rate constants were directly proportional to the flavin concentration

(6) Procedure adapted from that of: Bruice, T. C.; Chan, T. W.; Taulane, J. P.; Yokoe, I.; Elliot, D. L.; Williams, R. F.; Novak, M. *J. Am. Chem. Soc.* **1977**, *99*, 6713–6720.

(7) Satisfactory combustion analysis was obtained.

(8) Funk, M. O.; Nakagawa, Y.; Skochdopole, J.; Kaiser, E. T. *Int. J. Peptide Protein Res.* **1979**, *13*, 296–303.

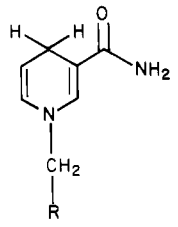
(9) An assay measuring the cleavage of *N*-benzoyl-DL-arginine-*p*-nitroanilide was used as was described in ref 2.

(10) The N^1-alkyl-1,4-dihydronicotinamides were made by the reduction of the corresponding N^1-alkylnicotinamide halides by sodium dithionite in carbonate buffer, as described for **10**: Suelter, C. H.; Metzler, D. C. *Biochim. Biophys. Acta* **1960**, *44*, 23–33.

(11) This program for the estimation of parameters by nonlinear regression was obtained from B. A. Blumenstein, Emory University.

(12) Initial rates were fit to a hyperbolic V vs [S] plot by using the iterative curve-fitting computer program obtained from John Westley, Department of Biochemistry, The University of Chicago, following the methodology of Wilkinson: Wilkinson, G. N. *Biochem. J.* **1961**, *80*, 324–332.

(13) Agreement between these kinetic parameters is achieved only when the reactions are conducted in the presence of superoxide dismutase and catalase. The N^1-alkyl-1,4-dihydronicotinamides are known to be sensitive to free radical chain oxidations initiated by superoxide anion. See: Porter, D. J. T.; Bright, H. J. *J. Biol. Chem.* **1980**, *255*, 7362–7370.



9, R = C_6H_5
10, R = CH_2CH_3
11, R = $(CH_2)_4CH_3$

and extrapolate to zero as the concentrations of flavin approaches zero.[14,15] These model rate constants are also shown in Table I. The data are consistent with the expected mechanism of oxidation: a bimolecular oxidation of nicotinamide by flavin followed by a fast reoxidation of the reduced flavin by oxygen.

As can be seen from the data presented in Table I, flavopapain **1** is capable of producing enzymatic rate enhancements of about 600-fold by using N^1-hexyldihydronicotinamide **11** as a substrate. The next best semisynthetic enzyme, flavopapain **4**, is capable of producing only an 18-fold enzymatic rate enhancement for the same substrate.[3] Catalysis by flavopapain **1** thus represents an improvement of well over an order of magnitude in the rate enhancement.

The new flavopapain **1** is also more selective than is either the isolated flavin **3** or the previously described flavopapain **4**. The selectivity of enzyme **1** is in complete accord with our postualated hydrophobic binding interaction. Since the largest kinetic differences among the substrates reside in the K_m term, we feel that the selectivity of this enzyme may be derived mainly from differences in the binding ability for the various substrates. The K_m and k_{cat} may not be kinetically simple, and therefore further explanation of the catalytic efficiency and selectivity of this enzyme must await future mechanistic study.

The kinetic parameters determined for flavopapain **1** compare favorably with those for many natural flavoenzymes which oxidize dihydropyridine nucleotides. Although out best value of k_{cat}/K_m for **1** is well below the $k_{cat}/K_m \simeq 10^8$ M^{-1} s^{-1} reported for bovine heart NADH dehydrogenase,[16] this is an unusually efficient catalyst and the rate parameter measured probably represents an upper limit for this type of dehydrogenation reaction. Values of k_{cat}/K_m of 3227 M^{-1} s^{-1} [17] and 610 M^{-1} s^{-1} [18] have been reported for the oxidation of NADH by old yellow enzyme. Using the more recently obtained value of 610 M^{-1} s^{-1}, this represents a catalytic rate enhancement of 1365-fold over the rate of oxidation of NADH by lumiflavin—a rate enhancement on the same order as that which we have obtained. Old yellow enzyme will also oxidize N^1-propyl-1,4-dihydronicotinamide **9** with k_{cat}/K_m = 20 300 M^{-1} s^{-1}; this represents a rate enhancement of about 200 over that measured using lumiflavin. For this substrate, at least, our semisynthetic enzyme, with a k_{cat}/K_m of 59 000 M^{-1} s^{-1} is the superior catalyst.

The comparison between old yellow enzyme and flavopapain may not be the best one to make because the in vivo function of old yellow enzyme, as well as the identity of its natural substrates, remain unknown. Consequently, we have sought other examples of flavoenzymes with which to compare the kinetic parameters of flavopapain. One such enzyme is melilotate hydroxylase, a microbial monooxygenase which uses NADH and O_2 as substrates to hydroxylate melilotate and thus produces 2,3-dihydroxy-β-phenylpropionate and water. Extensive kinetic studies have revealed that enzyme first binds melilotate to form an oxidized enzyme–substrate complex, which reacts very rapidly with NADH forming a ternary complex of reduced flavoenzyme, NAD^+, and melilotate with an observed second-order rate constant of 2.3 × 10^6 M^{-1} s^{-1}.[19] This value is approximately 4 times larger than the k_{cat}/K_m value for the reaction of flavopapain **1** with N^1-hexyldihydronicotinamide.[20] Another useful comparison is to the glucose oxidase from *Aspergillus niger*. Values of k_{cat}/K_m of about 10 500 M^{-1} s^{-1} have been determined for this enzyme[21] which is thought to operate via a kinetic scheme analogous to the one we have postulated for flavopapain **1**. Here, flavopapain **1** is more effective in catalyzing the oxidation of a good substrate by a factor of over 50-fold. Clearly, the k_{cat}/K_m values measured for our semisynthetic flavopapain **1** are of roughly the same magnitude as those observed for typical naturally occurring flavoenzymes.

Further work, directed at elucidating the mechanism and stereospecificity of this interesting semisynthetic flavopapain **1** is now in progress.

Acknowledgment. Support of this research by NSF Grant DAR 7910245 (E.T.K.) and USPHS Postdoctoral Fellowship GM-07766-03 (J.T.S.) is gratefully acknowledged.

(14) These results are obtained only when kinetics are measured in the presence of superoxide dismutase and catalase.
(15) Although the oxidation of dihydronicotinamide by flavins has been postulated to proceed via a charge-transfer complex, the dissociation constant for the complex of 0.1 M would mean that under the conditions of our experiments where [N^1-RNH] ≤ 2.5 × 10^{-4} M complex formation would be negligible and saturation would not be observed. See: Blankenhorn, G. *Biochemistry* **1975**, *14*, 3172–3176 and references therein.
(16) Singer, T. P. In *Biological Oxidations*: Singer, T. P., Ed.; Interscience Publishers: New York, 1968; pp 339–377.
(17) Honma, T.; Ogura, Y. *Biochim. Biophys. Acta* **1977**, *484*, 9–23.
(18) See references in ref 13.
(19) Massey, V.; Hemmerich, P. *Enzymes, 3rd Ed.* **1975**, *12*, 217–221.
(20) We thank one of the referees for suggesting this comparison.
(21) Gibson, Q. H.; Swoboda, B. E. P.; Massey, V. *J. Biol. Chem.* **1964**, *239*, 3927–3934.

A Strategy for the Synthesis of Cylindrical Macropolycyclic Hosts with Hydrophilic Interior Surfaces: Crown Ether Rings Fused by the Tetrahydroxymethylethylene (THYME) Unit[1]

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Host–guest chemistry,[2] the design, preparation, and study of organic compounds capable of molecular recognition in complexation, has recently become an exciting and rapidly growing area of research. Many elegant excursions into this field are described in the literature,[2-5] and great strides have been made toward development of methods for design and synthesis of host molecules and toward an understanding of the complexation process. In an effort to extend the host–guest frontier, we have begun a program directed toward the synthesis and study of cylindrical macropolycyclic hosts composed of crown ether rings fused by the tetrahydroxymethylethylene (THYME) unit. Consideration of prior art in the field and examination of CPK molecular models indicate that such hosts may have hydrophilic interior surfaces and, therefore, may show novel and interesting properties including useful catalytic activity. Herein, we report on successful completion of the first phase of this study: development of an efficient, regioselective strategy for synthesis of polycyclic cylindrical hosts possessing the THYME unit as the

[†]University of Colorado Chemistry X-ray facility.
(1) A preliminary account of some of this work was presented at the Second Symposium on Macrocyclic Compounds, Aug 14–16, 1978, at Brigham Young University, Provo, UT.
(2) (a) Cram, D. J.; Cram, J. M. *Science (Washington, D.C.)* **1974**, *183*, 803. (b) Cram, D. J.; Cram, J. M. *Acc. Chem. Res.* **1978**, *11*, 8–14.
(3) "Synthetic Multidentate Macrocyclic Compounds", Izatt, R. M., Christensen, J. J., Eds.; Academic Press: New York, 1978.
(4) (a) Lehn, J. M. *Struct. Bonding (Berlin)* **1973**, *16*, 1–69 (b) *Pure Appl. Chem.* **1977**, *49*, 857. (c) *Acc. Chem. Res.* **1978**, *11*, 49–57.
(5) (a) Bender, M. L.; Komiyama, M. "Cyclodextrin Chemistry"; Springer-Verlag: New York, 1977. (b) Bender, M. L.; Komiyama, M. "Bioorganic Chemistry", van Tamelen, E. E., Ed.; Academic Press: New York, 1977; Vol. 1, Chapter 2.

0002-7863/81/1503-6213$01.25/0 © 1981 American Chemical Society