# Semisynthetic Enzymes: Characterization of Isomeric Flavopapains with Widely Different Catalytic Efficiencies

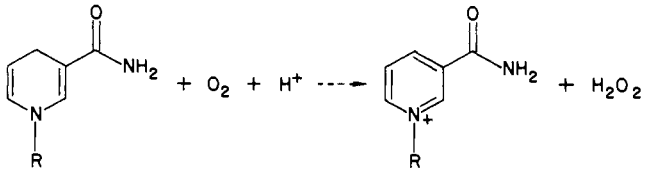
**James T. Slama,† Czestaw Radziejewski, SubbaRao Oruganti, and E. T. Kaiser***

*Contribution from the Department of Chemistry, The University of Chicago, Chicago, Illinois 60637, and The Laboratory of Bioorganic Chemistry and Biochemistry, The Rockefeller University, New York, New York 10021.* *Received March 12, 1984*

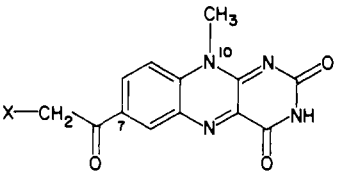
**Abstract:** Flavopapain **6** has been prepared by alkylation of the active site cysteine-25 of papain with 8α-(bromoacetyl)-10-methylisoalloxazine. This semisynthetic enzyme was shown to serve as a catalyst for the oxidation of dihydronicotinamides, exhibiting saturation kinetics and up to 600-fold rate accelerations relative to a model flavin. This is contrasted to the behavior of flavopapain **9**, the product of the modification of papain with 6α-(bromoacetyl)-10-methylisoalloxazine. In this case no catalytic rate enhancement compared to the behavior of a model compound is observed. Since the two isoalloxazines are isomeric, the differences in the activities of the semisynthetic enzymes are interpreted in terms of differences in the geometry of the flavin at the active site. It is also noted that flavopapain **6** can exhibit some chiral discrimination toward optically active dihydronicotinamides.

A major goal of current research in the area of biomimetic chemistry is the design of catalysts that simulate the behavior of enzymes. Several approaches to catalyst design have been based upon the premise that a substrate binding site can be transformed into a specific biomimetic catalyst. A binding cavity with definable specificity can be modified to introduce reactive functional groups within or adjacent to the binding site. Molecules that form inclusion complexes with the binding cavity are brought into close spatial proximity with these reactive groups and react with them, either stoichiometrically or catalytically, at greatly accelerated rates. A highly stereoselective reaction can be observed due to the defined geometry forced upon the transition state by the binding interaction. Cyclodextrins, which possess a hydrophobic binding cavity capable of forming inclusion complexes with a variety of organic molecules, have been chemically modified and successfully exploited as biomimetic catalysts.[1] Alternatively, chemically modified synthetic polymers have been used successfully in biomimetic reactions as well.[2]

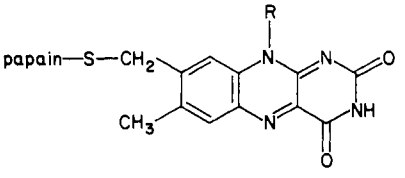
We have embarked on a different approach to catalyst design, that of modifying an existing enzyme at or near its binding site with a reactive coenzyme analogue. If the enzymatic binding site remains intact, we expect that this process of "chemical mutation" should produce a semisynthetic enzyme which will combine the characteristic chemical reactivity of the coenzyme with the substrate specificity of the parent enzyme. Previous work in this laboratory has centered on studies of a series of flavopapains,[3-9] a family of semisynthetic enzymes in which reactive flavin analogues (i.e., isoalloxazine derivatives) are attached to the active site cysteine-25 of the simple hydrolytic enzyme papain [E.C. 3.4.4.10]. A suitably positioned flavin can then take advantage of the adjacent hydrophobic binding groove which has been demonstrated to be a part of the papain active site.[10] These semisynthetic enzymes have been found to be effective redox catalysts for the oxidation of 1-alkyl-1,4-dihydronicotinamides, according to



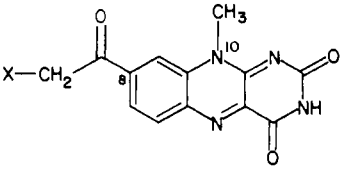
Alkylation of the active site cysteine-25 of papain with the flavin analogue 7α-(bromoacetyl)-10-methylisoalloxazine (**1**) has been demonstrated to yield a flavopapain **2** which is an effective oxidoreductase. That is, flavopapain **2** was found to catalyze the oxidation of 1-alkyldihydronicotinamides with rates that are as high as 20 times greater than were the rates of oxidation of the



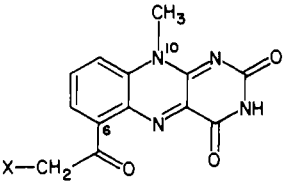
**1**, X=Br
**2**, X=papain Cys$^{25}$-S
**3**, X=H



**4**, R=$CH_2(CHOAc)_3CH_2OAc$
**5**, R=$CH_3$



**6**, X=papain-S
**7**, X=Br
**8**, X=H



**9**, X=papain-S
**10**, X=Br
**11**, X=H

† Current address: Department of Biochemistry, The University of Texas Health Science Center, San Antonio, Texas 78284.

* Address reprint requests and correspondence to this author at The Laboratory of Bioorganic Chemistry and Biochemistry, The Rockefeller University, 1230 York Avenue, New York, New York 10021.

(1) Bender, M. L.; Komiyama, M. "Cyclodextrin Chemistry"; Springer-Verlag: Berlin, 1978. Tabushi, I. *Acc. Chem. Res.* **1982**, *15*, 66–72. Breslow, R. *Science (Washington, D.C.)* **1982**, *218*, 532–537.
(2) Shinkai, S.; Yamada, S.; Ando, R.; Kunitake, T. *Bioorg. Chem.* **1980**, *9*, 238–247.
(3) Levine, H. L.; Nagakawa, Y.; Kaiser, E. T. *Biochem. Biophys. Res. Commun.* **1977**, *76*, 64–70.
(4) Otsuki, T.; Nakagawa, Y.; Kaiser, E. T. *J. Chem. Soc., Chem. Commun.* **1978**, 457–459.
(5) Levine, H. L.; Kaiser, E. T. *J. Am. Chem. Soc.* **1978**, *100*, 7670–7677.
(6) Levine, H. L.; Kaiser, E. T. *J. Am. Chem. Soc.* **1980**, *102*, 343–345.
(7) Kaiser, E. T.; Levine, H. L.; Otsuki, T.; Fried, H. E.; Dupeyre, R.-M. in "Biomimetic Chemistry"; Dolphin, D., McKenna, C., Murakami, Y., Tabushi, I., Ed.; American Chemical Society: Washington, DC, 1980; Adv. Chem. Ser. No. 191, pp 35–48.
(8) Fried, H. E.; Kaiser, E. T. *J. Am. Chem. Soc.* **1981**, *103*, 182–184.
(9) A preliminary account of this work has been published: Slama, J. T.; Oruganti, S. R.; Kaiser, E. T. *J. Am. Chem. Soc.* **1981**, *103*, 6211–6213.
(10) Berger, A.; Schechter, I. *Philos. Trans. R. Soc. London, Ser. B:* **1970**, *257*, 249–264.

0002-7863/84/1506-6778$01.50/0 

same substrates using the unsubstituted flavin **3**.[5] Also, under conditions where the concentrations of the dihydronicotinamides were in excess, the oxidation of the dihydronicotinamides as well as NADH by flavopapain **2** was found to exhibit saturation kinetics, with values of $K_m$ on the order of $10^{-4}$ M.

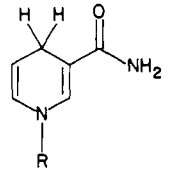
Not all flavopapains, however, have been found to be effective semisynthetic enzymes. Flavopapain **4** produced by alkylation of cys-25 with 8$\alpha$-bromo-2′,3′,4′,5′-tetra-*O*-acetylriboflavin and flavopapin **5**, the product of a similar modification using 8$\alpha$-bromolumiflavin, have been found to catalyze the oxidation of dihydronicotinamides only slightly faster (i.e., 3 times) than the rates achieved by using the corresponding unsubstituted flavins.[3,5,7] Additionally, unlike flavopapain **2**, flavopapains **4** and **5** failed to exhibit saturation kinetics.

The difference between these two classes of flavopapains had been anticipated from model building based upon the published X-ray diffraction studies of papain[11,12] as well as the published structures of several papain–inhibitor complexes.[13] The success of flavopapain **2** is attributed to the ability of the 7-acetyl carbonyl of the isoalloxazine to hydrogen bond with the backbone NH of Cys-25 and the $NH_2$ of Glu-19. Model building indicated that if such a hydrogen-bond contact is made, the rigid flavin ring is constrained to occupy a defined position along the surface of the papain molecule. It was also clear that when the flavin was positioned in this way, the dihydronicotinamide substrates could be positioned so that the dihydronicotinamide ring could lie adjacent to the flavin ring and, at the same time, the hydrophobic 1-alkyl tail could occupy the hydrophobic papain substrate binding site.

The isoalloxazine rings of flavopapain **4** and **5**, without this hydrogen bonding interaction, are not so constrained and are free to adopt conformations which place the flavin ring far enough away from the bound substrate that hydride transfer is no longer facilitated.

Further model building using the isomeric 8-acetyl-10-methylisoalloxazine attached via Cys-25 as in **6** and constrained by requiring the 8-acetyl carbonyl to hydrogen bond to Cys-25 and Glu-19 led to the prediction that 1-benzyl-1,4-dihydronicotinamide could fit into the binding site of the enzyme with the nicotinamide ring in close proximity to the flavin. Therefore, a prediction was made that flavopapain **6** should exhibit saturation kinetics and should produce relatively high catalytic rate enhancements. On the other hand, in the case of flavopapain **9** hydrogen bonding of the 6-acetyl carbonyl would require that the isoalloxazine ring move away from the substrate binding site. Hence, the prediction that flavopapain **9** would fall into the group of semisynthetic enzymes like **4** and **5** which exhibit low catalytic rate enhancements.

In order to test these predictions further, a synthesis of the requisite 8-acetyl-10-methylisoalloxazine **8** and the isomeric 6-acetyl-10-methylisoalloxazine **11** was devised, and the corresponding flavopapains **6** and **9** were produced. In the present article, we describe the preparation of these model enzymes, and we characterize their ability to oxidize the dihydronicotinamide substrates **12**–**14**.[9] Comparison of the activities of the isomeric flavopapains **2**, **6**, and **9** should provide us with a means to evaluate the subtle geometric requirements of our semisynthetic active site.




**12**, R=$CH_2CH_2CH_3$ (NPrNH)
**13**, R=$CH_2(CH_2)_4CH_3$ (NHxNH)
**14**, R=$CH_2Ph$ (NBzNH)
**15**, R=ADPR (NADH)

## Experimental Section

**General.** Proton magnetic resonance ($^1$H NMR) spectra were determined at 60 (Varian HA-60A), 270 (Brucker HS-270), or 500 mHz. Chemical shifts are expressed as $\delta$ values (in ppm) relative to an internal $Me_4Si$ standard for organic solvents and relative to an internal standard of DDS for aqueous solutions. Spectrophotometric determinations were performed on Beckman Acta MVI and Varian Cary 219 spectrophotometers equipped with thermostatic cell compartments and at a temperature of 25 °C. Melting points were determined on a Thomas-Hoover capillary melting point apparatus and are uncorrected. Elemental analyses were performed by the Analytical Laboratory, Department of Chemistry, University of California, Berkeley, or by Galbraith Laboratories, Knoxville, TN.

**Materials.** Except as noted, materials were obtained commercially and used without further purification. Papain was purchased as a suspension of recrystallized material in 0.05 M acetate, pH 5.0, from the Sigma Chemical Co. (St. Louis, MO) or from Worthington Biochemicals (Freehold, NJ). Buffers were prepared from distilled water which was passed through a demineralization unit (Continental Demineralizer).

**Purification and Assay of Papain.** Commercial preparations of papain were purified by affinity chromatography on a column of Gly-Gly-Tyr-(OBz)-Arg-agarose which was purchased from Miles Laboratories Research Product Division (Elkhart, IN), and the activity was determined spectrophotometrically by measuring the rate of hydrolysis of *N*-benzoyl-D,L-arginine-*p*-nitroanilide (BAPA), as has been described previously.[5]

**Modification of Papain with 8$\alpha$-(Bromoacetyl)-10-methylisoalloxazine.** Freshly purified papain in deionized water was treated with a 5-fold excess of the brominated flavin derivative **7**, following the procedure described previously.[5,9]

The concentrations of solutions of the modified flavopapain **6** were determined spectrophotometrically assuming $\epsilon_{442}$ $1.03 \times 10^4$ $M^{-1}$ $cm^{-1}$ (i.e., using $\epsilon_{442}$ for **8**).

The stoichiometry of the modification reaction was determined by calculating the amount of flavin present, using the absorbance at 442 nm, and employing the extinction given above. The amount of protein was calculated from the absorbance at 280 nm, using the molar extinction coefficient of papain ($\epsilon_{280}^{molar}$ 57 500[5]), after subtracting the contribution to the absorbance from the flavin ($A_{280}/A_{442}$ = 1.58 for flavin **8**).

**Modification of Papain with 6$\alpha$-(Bromoacetyl)-10-methylisoalloxazine.** Modification of papain with the brominated flavin derivative **10** was performed by using the same procedure as that used for the 8$\alpha$-bromoacetyl derivative **7**. Assuming that the spectrum of the flavin chromophore was unaffected by its attachment to the protein, the enzyme/flavin ratio in the flavopapain **9** was calculated to be 1.36.

**1-Benzyl-1,4-dihydronicotinamide (14)** was synthesized as described by Mauzerall and Westheimer.[14] Product was recrystallized from ethanol and water resulting in the isolation of yellow needles, mp 110.5–115 °C dec (lit.[14] 120–122 °C), electronic absorption spectrum (ethanol) $\lambda_{max}$ ($\epsilon$, $M^{-1}$ $L^{-1}$) 354 ($6.61 \times 10^{+3}$), lit.[14] 355 nm ($7.24 \times 10^{+3}$).

**1-Hexylnicotinamide Bromide (13).** Nicotinamide (12.2 g, 0.1 mol) was treated with 1-bromohexane (21 mL, 24.8 g 0.15 mol) as a solution in methyl ethyl ketone (100 mL) and at reflux for 72 h. The white crystals which formed were collected on a Buchner funnel, washed with several portions of reagent grade acetone, and dried, resulting in the isolation of 25.9 g (90% theory) of crystalline product, mp 200–201.5 °C.

**1-Hexyl-1,4-dihydronicotinamide (13).** A three-neck 250-mL round-bottom flask, fitted with an overhead stirrer and an $N_2$ bubbler, was charged with 1-hexylnicotinamide bromide (3.07 g; 0.011 mol), sodium carbonate (7.5 g, 0.071 mol), and water (60 mL). When a solution had formed, toluene (50 mL) was added and the two-phase mixture stirred rapidly for 10 min as a vigorous stream of $N_2$ was bubbled through the mixture. This mixture was protected from light and treated with sodium dithionite ($Na_2S_2O_4$, 7 g, 0.04 mol), added in portions over 4 min. The reaction was allowed to stand for an additional 6 min, at which time stirring was stopped and the phases separated. The aqueous phase was washed 3 times with 30-mL portions of toluene. The organic phases were combined, dried above 10 g of anhydrous sodium sulfate, and filtered, and the solvent was removed in vacuo to leave a residue weighing 2.67 g.

This solid could be recrystallized by dissolving in 100 mL of warm (50 °C) diisopropyl ether (previously deoxygenated by bubbling $N_2$ through the solvent), removing $\sim^1/_2$–$^2/_3$ of the solvent, and cooling by evapora-

(11) Drenth, J.; Jansonius, J. N.; Koebeck, R.; Swen, H. M.; Wolthers, B. G. *Nature (London)* **1968**, *218*, 929–931.
(12) Drenth, J.; Jansonius, J. N.; Koeboek, R.; Wolthers, B. G. *Adv. Protein Chem.* **1977**, *25*, 79–115.
(13) Drenth, J.; Kalk, K. H.; Swen, H. M. *Biochemistry* **1976**, *15*, 3731–3738.
(14) Mauzerall, D.; Westheimer, F. H. *J. Am. Chem. Soc.* **1954**, *77*, 2261–2264.

tion in vacuo (rotatory evaporator). Solid was collected, washed with cold diisopropyl ether, and dried in vacuo, resulting in the isolation of light yellow crystals: mp 63–65 °C; NMR ($CDCl_3$, 270 mHz) δ 0.88 (t, $J$ = 7 Hz, 3 H), 1.28 (s, 6 H), 1.51 (br t, 2 H), 3.07 (t, $J$ = 7 Hz, 2 H), 3.15 (d, $J$ = 1 Hz, 2 H), 4.71 (m, 1 H), 5.72 (d of d, $J$ = 1, 8 Hz, 1 H), 5.86 (br s, 2 H), 7.02 (s, 1 H).

Anal. Calcd for $C_{12}H_{20}N_2O$: C, 69.17; H, 9.68; N, 13.46. Found: C, 68.86; H, 9.52; N, 13.26.

**1-Propyl-1,4-dihydronicotinamide (12)** was synthesized from 1-propylnicotinamide iodide (mp 182–184 °C[15]) as described by Suelter and Metzler[16] and recrystallized from ethyl acetate to produce yellow crystals, mp 89–91.5 °C (lit.[16] 91–91.5 °C), electronic absorption spectrum (solvent 0.1 M Tris buffer, 1 mM EDTA pH 7.5) $\lambda_{max}$ (ε, $M^{-1}$ $cm^{-1}$) 362 ($7.53 \times 10^{+3}$) lit.[16] ($H_2O$) 360 nm ($7.06 \times 10^{+3}$).

**1-(2,4-Dinitrophenyl)-3-carbamoylpyridinium Tetrafluoroborate.** The procedure of Lettré et al.[17] for the synthesis and purification of the chloride salt was not found to be satisfactory in our hands. Consequently, it was modified as described below.

Nicotinamide (6.05 g, 0.05 mol) and 2,4-dinitrophenyl chloride (11.1 g, 0.055 mol, Aldrich, purified by crystallization) were dissolved in reagent grade methanol and refluxed for 30 h. The resulting solution was highly colored. Solvent was removed in vacuo, the residue dissolved in 50 mL of water, and this aqueous mixture extracted 8 times with 25-mL portions of chloroform. This extraction effectively removed the starting 2,4-dinitrophenyl chloride but not nicotinamide. Nicotinamide was removed by extracting the aqueous phase with chloroform in a continuous extraction apparatus for 48 h. After extraction, phases were separated and the organic layer was discarded. Water was removed in vacuo to leave an amorphous residue weighing 8.0 g (50% of theoretical). This residue, which consisted of the impure 1-(2,4-dinitrophenyl)-3-carbamoylpyridinium chloride could be isolated as a tan hygroscopic solid by precipitation from methanolic solutions using diethyl ether. Repeated attempts to crystallize this material as described by Lettré et al.[17] failed.

1-(2,4-dinitrophenyl)-3-carbamoylpyridinium tetrafluoroborate was prepared from this material by treating 7.37 g of the chloride, brought to a volume of 27 mL with water, with 4.2 g of sodium tetrafluoroborate (38 mmol) as a solution in 4–5 mL of water. An oily material formed, which was redissolved by heating on a steam bath and allowed to cool slowly. Solid (5.85 g) was collected, recrystallized from methanol (~2 g from ca. 50 mL) and dried in vacuo to yield colorless crystals, mp 167–169 °C.

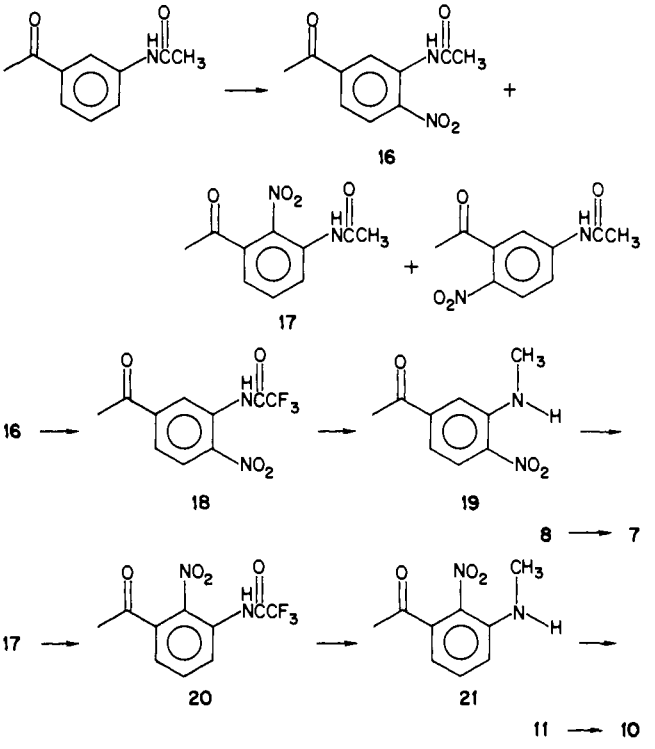
Anal. Calcd for $C_{12}H_9O_5N_4BF_4$: C, 38.33; H, 2.40; N, 14.90. Found: C, 38.22; H, 2.35; N, 14.77.

**1-(*d*-(+)-α-Methylbenzyl)-3-carbamoylpyridinium Tetrafluoroborate.** A stirred solution of *d*-(+)-α-methylbenzylamine (290 μL, 2.25 mmol, Aldrich, $[\alpha]^{23}$ +38° (neat)) in 5 mL of acetonitrile (distilled from above $P_2O_5$) was treated with a solution of 1-(2,4-dinitrophenyl)-3-carbamoylpyridinium tetrafluoroborate (5.64 mg, 1.5 mmol) dissolved in 5 mL of acetonitrile, added dropwise over a period of 5–10 min. After the addition was complete, the resultant solution was stirred at ambient temperature for 2 h. The reaction mixture was next poured into 50 mL of water, and the aqueous phase washed 5 times with 15-mL portions of methylene chloride to remove the 2,4-dinitroaniline. The organic washings were discarded, and the colorless aqueous solution was lyophilized leaving a colorless amorphous residue weighing 0.37 g (79% of theory): NMR (500 mHz, $D_2O$ and DSS standard) δ 9.36 (s, 1 H), 9.10 (d, $J$ = 7 Hz, 1 H), 8.90 (d of d, $J$ = 8 and 0.9 Hz, 1 H), 8.20 (t, $J$ = 7 Hz, 1 H), 7.54 (s, 5 H), 6.27 (q, $J$ = 7 Hz, 1 H), 2.15 (d, $J$ = 7 Hz, 3 H).

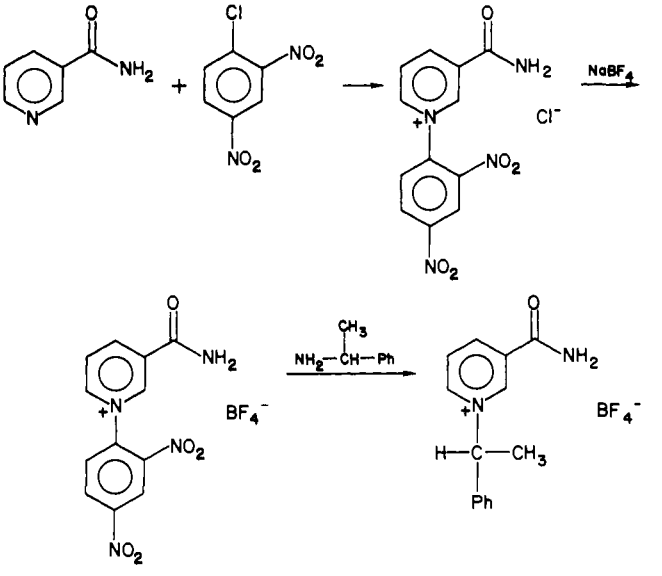
**1-(*l*-(−)-α-Methylbenzyl)-3-carbamoylpyridinium Tetrafluoroborate.** This compound was prepared and characterized as above except that *l*-(−)-α-methylbenzylamine (Aldrich, $[\alpha]^{20}$ −39° (neat)) was used as the starting amine.

**1-(*d*-(+)-α-Methylbenzyl)-1,4-dihydronicotinamide.** The 1-(*d*-(+)-α-methylbenzyl)-3-carbamoylpyridinium tetrafluoroborate (370 mg, 1.2 mmol) was dissolved in 10 mL of $H_2O$, sodium carbonate added (1 g) and, when this was dissolved, sodium dithionite (1 g) added. Nitrogen was bubbled through the mixture for 1 min; the flask was sealed and transferred to a refrigerator for $1^1/_2$ h. At the end of this time the aqueous mixture was extracted 5 times with 10-mL portions of methylene chloride, the combined organic washings were dried above 5 g of anhydrous sodium sulfate, the mixture was filtered, and the solvent was evaporated in vacuo to leave a yellow amorphous residue weighing 214 mg: NMR (500 mHz, $CD_2Cl_2$) δ 7.38–7.24 (m, 5 H), 7.12 (d, $J$ = 1–2 Hz, 1 H), 5.76 (d of d, $J$ = 8 and 1–2 Hz, 1 H), 5.29 (br, 1 H), 4.72 (m, 1 H), 4.43 (q, $J$ = 7 Hz, 1 H), 3.13 (m, 2 H), 1.57 (d, $J$ = 7 Hz, 3 H).

Scheme I. Synthesis of the Isoalloxazines




Scheme II. Synthesis of Nicotinamide Salts via the Zincke Reaction




**1-(*l*-(−)-α-Methylbenzyl)-1,4-dihydronicotinamide.** This compound was prepared and characterized as above except that 1-(*l*-(−)-α-methylbenzyl)-3-carbamoylpyridinium tetrafluoroborate was used as the starting material.

**(*R*)- and (*S*)-*N*-(α-Methylbenzyl)-1-alkyl-1,4-dihydronicotinamide.** These compounds were prepared according to the procedure of Ohno et al.[18] and characterized spectroscopically (NMR, optical rotation), starting from (*R*)-(+)-α-methylbenzylamine (Aldrich, $[\alpha]^{20}_D$ +38° (neat)) and (*S*)-(−)-α-methylbenzylamine (Aldrich, $[\alpha]^{20}_D$ −39° (neat)) as the optically active starting amines.

**Nitration of 3-Acetamidoacetophenone.** The nitration of 3-acetamidoacetophenone was carried out using a mixture of fuming nitric acid and acetic anhydride as has been described by Waters.[19] Products were

(15) Liao, S. Dissertation, University of Chicago, 1961, p 39.
(16) Suelter, C. H.; Metzler, D. E. *Biochim. Biophys. Acta* **1960**, *44*, 23–33.
(17) Lettré, H.; Haede, W.; Ruhbaum, E. *Justus Liebigs Ann. Chem.* **1953**, *579*, 123–132.
(18) Ohno, A.; Kimura, T.; Kim, S. G.; Yamamoto, H.; Oka, S.; Ohnishi, Y. *Bioorg. Chem.* **1977**, *6*, 21–29. Ohnishi, Y.; Kagami, M.; Ohno, A. *J. Am. Chem. Soc.* **1975**, *97*, 4766–4768.
(19) Waters, W. A. *J. Chem. Soc.* **1945**, 629.

separated chromatographically and further purified by crystallization. The 3-acetamido-4-nitroacetophenone (**16**) was crystallized from ethyl acetate/hexane to yield yellow plates, mp 121–123 °C (lit.[19] 121 °C). The identity of this compound was confirmed by conversion to 3-amino-4-nitroacetophenone by alkaline hydrolysis (KOH in ethanol/water) and crystallization from ethyl acetate/hexane, to yield dark crystals, mp 163–164 °C (lit.[19] 163 °C).

The isomeric 3-acetamido-2-nitroacetophenone (**17**) was similarly isolated as the major product from the nitration. It was crystallized from ethyl acetate and hexane to yield white needles, mp 166 °C (lit.[19] 165 °C). It could be converted upon hydrolysis (KOH in ethanol and water) to 3-amino-2-nitroacetophenone, mp 89.5–91.5 °C (lit.[19] 92 °C).

**4-Nitro-3-(trifluoroacetamido)acetophenone (18).** The 3-amino-4-nitroacetophenone (1.2 g, 6.6 mmol) was dissolved in 3.3 mL of trifluoroacetic acid (reagent grade, distilled), cooled in an ice/water bath, and treated with 1.1 mL of trifluoroacetic anhydride (reagent grade, distilled from $P_2O_5$). The resulting solution was allowed to stir for 25 min, at which time the mixture was poured into 100 mL of cold water. This mixture was extracted 4 times with 25-mL portions of chloroform, the organic extracts were dried above sodium sulfate, and the solvent was removed in vacuo, to yield 1.79 g (98% theory). This material was crystallized from chloroform/hexane and further purified by sublimation (bath temperature 85–95 °C, 10 $\mu$Hg of pressure) to produce a yellow solid: mp 104–106 °C; $R_f$ (silica gel, $CHCl_3$/EtOAc, 5:1) 0.67; NMR (60 mHz, $CDCl_3$) $\delta$ 2.65 (s, 3 H), 7.83 (dd, $J$ = 8.75 and 2 Hz, 1 H), 8.33 (d, $J$ = 8.75 Hz, 1 H), 9.18 (d, $J$ = 2 Hz, 1 H), 11.17 (br, 1 H).

Anal. Calcd for $C_{10}H_7F_3N_2O_4$: C, 43.49; H, 2.55; N, 10.14. Found: C, 43.52; H, 2.61; N, 10.18.

**3-(Methylamino)-4-nitroacetophenone (19).** 4-Nitro-3-(trifluoroacetamido)acetophenone (1.20 g, 4.35 mmol, purified by sublimation) was dissolved in ca. 7–8 mL of dimethoxyethane (freshly distilled from above excess $LiAlH_4$). The stirred solution was treated with 2.5 g of anhydrous $K_2CO_3$ (dried by heating at 300 °C for 8 h under high vacuum), followed by 0.7 mL of methyl iodide (free from $I_2$, purified by distillation from above $P_2O_5$). The reaction mixture was protected from water with either a drying tube or a $N_2$ blanket and allowed to stir at ambient temperature overnight. Water (50 mL) and ethanol (50 mL) were added in order to obtain an alkaline solution (with heating on a steam bath for a few minutes) in which hydrolysis of the trifluoroacetyl protecting group would occur. Product was extracted into chloroform, the organic extracts were dried above anhydrous sodium sulfate, and the solvent was removed to give 0.87 g (quantitative yield) of a red-orange solid. This could be crystallized from ethyl acetate/hexane to yield short red crystals: mp 110.5–111 °C (alternatively, crystallization can be from water and ethanol, to yield crystals melting at 105–106 °C after drying); NMR ($CDCl_3$, 60 mHz) $\delta$ 2.61 (s, 3 H), 3.08 (d, $J$ = 5 Hz, 3 H), 7.12 (dd, $J$ = 2 and 9 Hz, 1 H), 7.4 (d, $J$ = 2 Hz, 1 H), 8.21 (d, $J$ = 9 Hz, 1 H), 7.93 (br, 1 H).

Anal. Calcd for $C_9H_{10}N_2O_3$: C, 55.66; H, 5.19; N, 14.43. Found: C, 55.81; H, 5.37; N, 14.50.

**8-Acetyl-10-methylisoalloxazine (8).** A solution of 3-(methylamino)-4-nitroacetophenone (0.45 g, 2.3 mmol) was dissolved in ethanol (75 mL) and 105 mg of 10% palladium on charcoal (Matheson, Coleman, and Bell) was added. The solution was deoxygenated by bubbling $N_2$ through it for 10–15 min then treated with $H_2$. Progress of the reduction was monitored by TLC (silica gel, $CHCl_3$/EtOAc, 5:1), and the reaction was stopped when starting material was consumed, usually within 45–60 min. Catalyst was removed by filtration, the precipitate washed with 15 mL of ethanol, and the combined ethanolic solution used immediately in the next reaction.

The solution of 3-(methylamino)-4-aminoacetophenone in 90 mL of ethanol was added to a solution of alloxan monohydrate (0.373 g, 2.33 mmol, Eastman Organic Chemicals, finely powdered) in 4.6 mL of concentrated hydrochloric acid. The resulting mixture was refluxed for 20 min then cooled in an ice bath and the solid recovered by filtration. The orange solid thus obtained was washed with ethanol and diethyl ether and dried to yield 270 mg. Another 48 mg of product separated from the filtrate on standing for a day. The overall yield of flavin was 318 mg (1.18 mmol, 51% theory).

The crude 8-acetyl-10-methylisoalloxazine was purified by washing with a small amount of hot $Me_2SO$, followed by several washes with dry diethyl ether and drying under reduced pressure (1 mmHg at 80 °C). The result was an orange solid: mp >300 °C; NMR (60 mHz, $CF_3CO_2D$) 3.11 (s, 3 H), 4.83 (s, 3 H), 9.0 (m, 2 H), 9.28 (m, 2 H); electronic absorption spectrum $\lambda_{max}$ ($\epsilon$, $M^{-1}$ $cm^{-1}$) (50 mM phosphate, pH 7.0; 1% $Me_2SO$), 442 (10 300), 341 (9450), 244 nm (26 100).

Anal. Calcd for $C_{13}H_{10}N_4O_3$: C, 57.78; H, 3.73; N, 20.73. Found: C, 57.86; H, 3.84; N, 20.70.

**8$\alpha$-(Bromoacetyl)-10-methylisoalloxazine Hydrobromide (7).** 8-Acetyl-10-methylisoalloxazine (20 mg, 0.074 mmol) was suspended in 25 mL of glacial acetic acid, which was heated in a 90 °C oil bath, and a solution of $Br_2$ in acetic acid (2.84 mL of 0.031 M $Br_2$ solution, 0.088 mmol of $Br_2$) was added dropwise over about 10 min. The solution was stirred at 90 °C for an additional 80 min and left to stand at room temperature overnight. Suspended starting material dissolved during the addition of $Br_2$, and subsequently product precipitated from solution as yellow crystals. Crystals were filtered, washed with diethyl ether, and dried overnight under reduced pressure to yield 23 mg (0.066 mmol, 67% theory), NMR (60 mHz, $CF_3COOD$) $\delta$ 4.91 (br s, 3 H), 4.97 (s, 2 H), 9.11 (m, 2 H), 9.41 (m, 1 H).

**2-Nitro-3-(trifluoroacetamido)acetophenone (20).** 3-Amino-2-nitroacetophenone (1.45 g, 8.1 mmol) was dissolved in 3.0 mL of distilled trifluoroacetic acid, stirred, and cooled in an ice bath. Trifluoroacetic anhydride (1.5 mL, distilled from above $P_2O_5$) was added, the mixture was protected from water with a drying tube and stirred for 20–25 min. The reaction mixture was next poured onto 125 mL of cold water and extracted 3 times with 25-mL portions of chloroform. Combined organic extracts were dried above sodium sulfate, and the solvent was removed. The product was crystallized from $CHCl_3$ (15 mL) and *n*-hexane (50 mL), resulting in the isolation of 1.90 g (85% theory) of white needles: mp 89–91 °C; NMR (60 mHz, $CDCl_3$) $\delta$ 2.73 (s, 3 H), 7.67 (dd, $J$ = 8 and 1.5 Hz, 1 H), 8.05 (dd, $J$ = 8 and 1.5 Hz, 1 H), 8.77 (dd, $J$ = 8.5 and 1.5 Hz, 1 H), 10.7 (br, 1 H).

Anal. Calcd for $C_{10}H_7F_3N_2O_4$: C, 43.49; H, 2.55; N, 10.14. Found: C, 43.45; H, 2.63; N, 10.01.

**3-(Methylamino)-2-nitroacetophenone (21).** Methylation was accomplished by using the procedure of T. Bruice et al.[20] 2-Nitro-3-(trifluoroacetamido)acetophenone (1 g, 3.62 mmol) was treated with methyl iodide (2.74 g) and powdered KOH (0.45 g, 85%) in 10 mL of acetone (taken from a freshly opened bottle, reagent grade). The mixture was refluxed for 30 min, cooled to room temperature, and filtered. The residue obtained after removing volatiles from the filtrate was purified by passing it through a short column of neutral alumina and developing with benzene (any remaining (trifluoroacetyl)amine was deprotected at this time). The product was recrystallized from ethyl acetate/hexane to yield 0.647 g (92% of theory) of orange needles: mp 86–86.5 °C; NMR (60 mHz; $CHCl_3$), $\delta$ 2.48 (s, 3 H), 3.07 (d, $J$ = 5.4 Hz, 3 H), 6.58 (dd, $J$ = 1.5 and 7 Hz, 1 H), 6.99 (dd, $J$ = 1.5 and 8.5 Hz, 1 H), 7.50 (dd, $J$ = 7 and 8.5 Hz, 1 H), 8.09 (br, 1 H).

Anal. Calcd for $C_9H_{10}N_2O_3$: C, 55.66; H, 5.19; N, 14.43. Found: C, 55.81; H, 5.37; N, 14.50.

**6-Acetyl-10-methylisoalloxazine (11).** A solution of 3-methyl-2-nitroacetophenone (627 mg, 3.2 mmol) in 75 mL of absolute ethanol containing 125 mg of a 10% palladium on charcoal catalyst was deoxygenated by bubbling $N_2$ through the mixture for 2 h. Hydrogen gas was next introduced and the reduction conducted for 2 h by bubbling $H_2$ gas through the suspension. The reaction was monitored by TLC and stopped when starting material was consumed. The solution was filtered to remove the catalyst, and the precipitate was washed with 50 mL of ethanol.

The combined ethanolic extract was added to a solution of alloxan monohydrate (525 mg, 3.28 mmol, finely powdered) in 6.5 mL of concentrated HCl, and this mixture was heated on a steam bath to ca. 55–60 °C. A temperature of between 50 and 60 °C was maintained with intermittent heating for 70 min, at which time the solution was refluxed briefly (15 min). The reaction mixture was cooled in an ice/salt bath, and the greenish yellow insoluble product was removed by filtration and washed with quantities of ethanol and diethyl ether. This material was dried in vacuo, resulting in the isolation of 0.64 g (73% theory) of a green-yellow solid. Product crystallized from dimethyl sulfoxide (23 mL, at 150 °C) and was washed with ethanol and ether and dried in vacuo, resulting in the isolation of 498 mg of a yellow solid: mp >300 °C; NMR (500 mHz, $CF_3CO_2D$), $\delta$ 3.08 (s, 3 H), 4.67 (s, 3 H), 8.37 (m, 1 H), 8.51 (m, 2 H); electronic absorption spectrum $\lambda_{max}$ ($\epsilon$, $cm^{-1}$ $M^{-1}$) (50 mM Tris-HCl, pH 7.5, 1% $Me_2SO$), 433 ($1.075 \times 10^{+3}$), 340 ($7.58 \times 10^{+3}$), 263 nm ($3.26 \times 10^4$).

Anal. Calcd for $C_{13}H_{10}N_4O_3$: C, 57.78; H, 3.73; N, 20.73. Found: C, 57.61; H, 3.83; N, 20.72.

**6$\alpha$-(Bromoacetyl)-10-methylisoalloxazine Hydrobromide (10).** The 6-acetyl-10-methylisoalloxazine (100 mg) was suspended in 20 mL of hot acetic acid (90 °C) and treated with 7.5 mL of 0.057 M bromine in acetic acid, added dropwise over a period of 15 min. The resulting mixture was stirred at 90 °C for an additional 45 min and was allowed to cool and stir overnight at room temperature. The precipitate was collected, washed with ethyl ether, and dried in vacuo to yield 100 mg of a yellowish powder, NMR (500 mHz, $CF_3CO_2D$) $\delta$ 4.74 (s, 3 H), 4.98 (s, 2 H), 5.8–8.6 (m, 3 H).

(20) Bruice, T. C.; Chan, T. W.; Taulane, J. P.; Yokoe, I.; Elliott, D. L.; Williams, R. F.; Novak, M. *J. Am. Chem. Soc.* **1977**, *99*, 6713–6720.

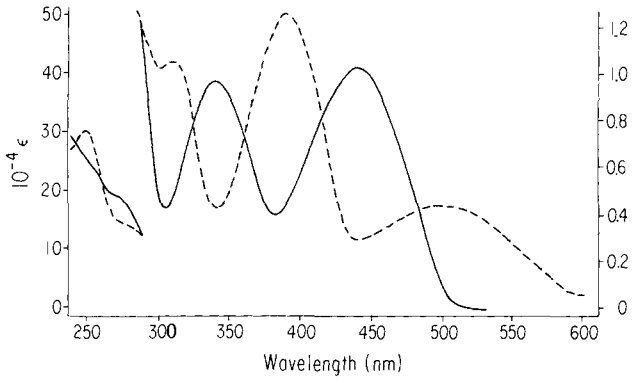



**Figure 1.** Electronic absorption spectrum of 8-acetyl-10-methylisoalloxazine, in 0.1 M Tris, 1 mM EDTA, pH 7.5. Spectrum of the reduced flavin is shown with a dashed line. Left-hand ordinate corresponds to extinction coefficients at wavelengths below about 290 nm. Right-hand ordinate corresponds to extinction coefficients at wavelengths above 290 nm.

**Kinetics of Model and Enzymatic Oxidations.** The rates of aerobic oxidation of the various dihydronicotinamides by model flavins **8** and **11** and the flavopapains were determined by measuring the decrease in the nicotinamide absorption which occurs between 340 and 360 nm. In a typical experiment, 3.0 mL of buffer (0.1 M Tris·HCl, 0.1 mM EDTA, pH 7.5) was added to a cuvette (4-mL volume, 1-cm path length) held in a thermostatic cell holder controlled at 25.0 °C. If desired, catalase (3500 units, 100 μg) and superoxide dismutase (10 μg) were added at this point. The dihydronicotinamide was introduced next, as an ethanolic solution (ca. $1.5 \times 10^{-2}$ M), and the reaction initiated by the addition of either the model flavin (as a solution in $Me_2SO$) or the flavopapain (as a solution in deionized water).

Concentrations of the dihydronicotinamide always exceeded that of the flavin by factors of between 20 and 200.

The enzymatic oxidation of 1-hexyl-1,4-dihydronicotinamide was examined at 27 °C by using a 5-cm path length cell.

## Results and Discussion

**Synthesis and Properties of Isoalloxazines 8 and 11.** The common starting material for both the 8- and the 6-acetyl-10-methylisoalloxazines was 3-acetamidoacetophenone. The nitration of 3-acetamidoacetophenone using a mixture of fuming nitric acid and acetic anhydride has been reported to yield a mixture of the 2-, 3-, and 6-nitro-3-acetoamidoacetophenones. Products, which were separated by crystallization, were unambiguously characterized by hydrolysis of the acetyl group and conversion of the resultant amines to known compounds by diazotization.[19]

In our hands, conversion of the chromatographically purified 3-acetamido-4-nitroacetophenone (**16**) to 8-acetyl-10-methylisoalloxazine (**8**) was accomplished in five steps and in 48% overall yield. The 6-acetyl-10-methylisoalloxazine (**11**) was produced by an analogous sequence with a comparable overall yield. Monobrominations of **8** and **11** were carried out using a stoichiometric amount of $Br_2$ in hot acetic acid, to yield the bromoacetyl derivatives **7** and **10**, respectively.

At pH 7.0, the main visible absorption band for 8-acetyl-10-methylisoalloxazine (**8**), illustrated in Figure 1, occurs at 442 nm, essentially unshifted when compared to lumiflavin ($\lambda_{max}$ 443) or to the electron-deficient 8-cyano-3,10-dimethylisoalloxazine ($\lambda_{max}$ 441).[20] The secondary peak of **8** occurs at 341 nm, and is of only slightly lower intensity than the primary peak ($\epsilon_{341}/\epsilon_{442} = 0.91$). This represents a blue shift of 19 nm from the secondary absorption of lumiflavin ($\lambda_{max}$ of 360). The blue shift is less, however, than that observed for the 8-cyano flavin in which the corresponding absorption exhibits a $\lambda_{max}$ of 330 nm.

Anaerobic solutions of the oxidized 8-acetyl-10-methylisoalloxazine (**8**) can be reduced to the dihydroflavin by a variety of techniques (photobleaching, dithionite reduction, dihydronicotinamides, etc.). Photobleaching could be accomplished by exposing a dilute anaerobic solution of **8** ($4 \times 10^{-5}$ M) in aqueous buffer containing EDTA (0.1 M Tris, 1 mM EDTA, pH 7.5) to an ordinary 100-W incandescent light for a few minutes. The spectrum of the dihydroflavin, shown in Figure 1, is characterized by absorption maxima at 496, 390, 312, 280 (shoulder), and 249 nm. Such an absorption spectrum is somewhat unusual, both when compared to normal and to the electron-deficient isoalloxazines. The reduced 8-cyanoisoalloxazine possesses a strong absorption band at 370 nm, a band not found in the spectra of dihydrolumiflavin. A corresponding band is observed at 390 nm in the spectrum of the reduced 8-acetyl flavin. However, while the reduced 8-cyanoisoalloxazine exhibits only a nondescript decreasing tail beyond 420 nm, reduced 8-acetyl-10-methylisoalloxazine exhibits a distinct well-formed absorption band with a $\lambda_{max}$ at 496 nm.

Upon aeration of an anaerobic solution of 8-acetyl-10-methylisoalloxazine the spectrum of the oxidized isoalloxazine is generated within a few seconds, demonstrating that the reduction is reversible.

A long-wavelength maxima at 505 nm was also observed in the spectrum of the reduced 6-acetyl-10-methylisoalloxazine (**11**). This absorption is assigned to the dihydroflavin rather than to the flavin semiquinone since EPR experiments failed to detect the presence of any free radical species in the colored (red) solutions of reduced acetyl flavin.

**Characterization of Flavopapain 6.** The modification of papain with 8α-(bromoacetyl)-10-methylisoalloxazine (**7**) and the characterization of the resultant flavopapain **6** as the 1:1 adduct of papain and the isoalloxazine, attached via a thioether linkage with cysteine-25, have already been described.[9] Briefly summarized, dilute solutions of papain in distilled water were treated with an excess of **7**, and the resultant decrease in the hydrolytic activity of papain was monitored to follow the course of the reaction, until either no further decrease of enzymatic activity could be observed or until less than 5% of the original hydrolytic activity was detected. The reaction was quenched with a stoichiometric amount of cysteine. If necessary, modification was repeated 1 or 2 more times. Excess flavin was removed by dialysis against distilled water.

The electronic absorption spectra of both the oxidized and the reduced forms of flavopapain **6** appear simply to be the result of the additive combination of the spectrum of papain and that of either the oxidized or the reduced isoalloxazine **8**. Further, the spectrum of flavopapain **6** does not change detectably when detergent (2% SDS) is added to the solution or upon heating the papain/detergent solution at 100 °C for 5 min.

It has been observed that modifications of papain with 8α-bromo-2′,3′,4′,5′-tetra-*O*-acetylriboflavin under aerobic conditions lead not to the expected **4** but to a species where the sulfur of cysteine-25 bridging the flavin to the enzyme is found to be oxidized, apparently to the sulfone oxidation state.[4] In view of the possibility of the formation of such compounds, an attempt was made to determine if the product of the reaction of papain with the (bromoacetyl)isoalloxazine **7** was indeed the flavopapain **6** with the sulfur in the sulfide oxidation state or if an oxidation had taken place during modification, resulting in a bridging sulfur present as a sulfoxide or sulfone. Treatment of flavopapain **6** with oxidizing agents ($H_2O_2$ or performic acid) did not lead to a change in the electronic absorption spectrum of the isoalloxazine chromophore. This might indicate either that the absorption spectrum is insensitive to changes in the oxidation state of sulfur, or that the sulfur is already present in the oxidized form, arising through oxidation during the aerobic modification.

An attempt was made to distinguish between these alternatives by carrying out the modification of papain with the reduced form of the (bromoacetyl)isoalloxazine **7** and under anaerobic conditions. Such an experiment was conducted entirely under an $N_2$ atmosphere and by using solutions that had been freed of $O_2$. Reduced **7** was prepared in situ just prior to its addition to the papain solution, by reduction using a 3–4-fold excess of 1-benzyl-1,4-dihydronicotinamide. Under such conditions, the modification was found to be very much less efficacious: even after an extended period of time (24–48 h) and using a significant excess of the (bromoacetyl)isoalloxazine **7** (10-fold excess) only low amounts of protein modification could be achieved (20–30% modification). This may represent a lower reactivity of the reduced

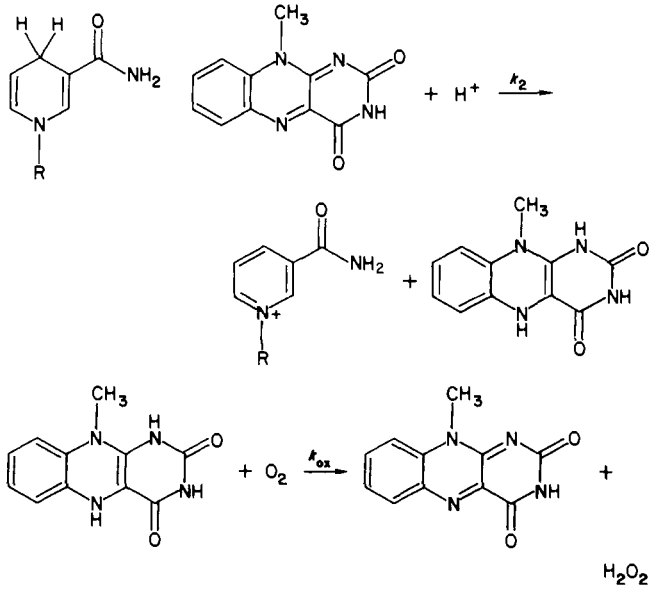
bromoacetyl compound toward papain, or it may be the result of a consumption of the alkylating agent in a side reaction not normally observed during aerobic modifications, such as reaction with the added reducing agent.

Nonetheless, a modest amount of flavopapain could be produced in this anaerobic modification. Although the isoalloxazine ring was present in far less than stoichiometric quantities, the UV–vis absorption spectrum of this material at wavelengths >340 nm was identical with that produced following the standard aerobic modification. Treatment of this anaerobically produced flavopapain with hydrogen peroxide (25 μL of 30% $H_2O_2$ in 3 mL) led to no detectable change in the isoalloxazine absorption in this region.

The fluorescent properties of flavopapain **6** and the parent isoalloxazine **8** were also compared. Relatively concentrated solutions of isoalloxazine **8** (ca. $10^{-5}$ M in $H_2O$ containing 0.2% $Me_2SO$) could be excited at 280 or 450 μm to produce a fluorescent emission spectrum with a maximum at 535 μm. A similar emission maximum was observed on the excitation of flavopapain **6**. The necessity for such highly concentrated solutions of isoalloxazine **8** or flavopapain **6** for observation of fluorescent spectra indicates that the fluorescence for the compounds is highly quenched when compared to lumiflavin, or even to 7-acetyl-10-methylisoalloxazine (**3**).

A comparison of the emission spectra of flavopapain **6** and the model compound **8** in which the concentrations of the isoalloxazine rings were roughly equivalent (that is, within a factor of 2) revealed that excitation at 450 nm produced the same structureless emission centered on 535 nm in both cases. The intensities of the emission, however, were quite different: the relative fluorescent intensity of flavopapain **6** is only 16% that of the parent 8-acetyl-10-methylisoalloxazine (**8**). The quenching of the emission of **6** relative to **8** is consistent with the presence of the thioether linkage rather than the sulfone.[4] On the basis of these results and by analogy to the isolation of flavopapain **2** by aerobic modification using isoalloxazine **1**, our expectation is that the structure of our new flavopapain is correctly written as **6**.

**Oxidation of Dihydronicotinamides by Model Flavins.** The nonenzymatic flavin-catalyzed oxidation of 1,4-dihydronicotinamides by oxygen to produce nicotinamides and $H_2O_2$ occurs readily at room temperature. The reaction is easily measured at neutral pH and in aqueous solutions spectrophotometrically by observing the decrease in the absorbance at 350–360 nm due to the dihydronicotinamide. The oxidation has been observed to be first order with respect to the flavin concentration as well as first order with respect to dihydronicotinamide concentration[16] and can be envisioned to proceed via the simple two step mechanism:




In oxygen-saturated aqueous solutions, $k_{ox} >> k_2$, and accumulation of the dihydroflavin intermediate or oxygen-limited rates of oxidation are usually not observed.[5,16,21] For flavins **3** and **11** oxygen limited oxidation rates have been seen at very high dihydronicotinamide concentrations.

**Table I.** Second-Order Rate Constants for Oxidation of Dihydronicotinamides by 8-Acetyl-10-methylisoalloxazine and 6-Acetyl-10-methylisoalloxazine[a]

| dihydronicotinamide | $k$, $M^{-1}$ $s^{-1}$ |
|---|---|
| 8-AcFl (**8**) | |
| NBzNH | 170 ± 2 |
| NPrNH | 878 ± 23 |
| NHxNH | 917 ± 18 |
| NADH | 5.12 |
| 6-AcFl (**11**) | |
| NBzNH | 64.3 |

[a] Measured at 25 °C in 0.1 M Tris·HCl, 0.1 mM EDTA, pH 7.5, 0–1% v/v $Me_2SO$. Each solution of 3.0 mL contains 10 units of superoxide dismutase and 3500 units of catalase except for the oxidation of NADH.

More recent kinetic studies of this reaction conclude that the oxidation process is considerably more complicated than is shown above. Oxidations of dihydronicotinamides by flavins are likely to proceed via a preequilibrium complex.[22] The dissociation constants of such complexes formed between 1-methyl-1,4-dihydronicotinamide and several representative flavins have been determined by fast reaction techniques and found to be in the vicinity of 0.1 M.[23,24] In the present study, concentrations of nicotinamide well below this value were always used, and therefore saturation due to complex formation should not be observed. The reaction under our conditions is expected to be first order with respect to dihydronicotinamide.

In our hands the oxidation reactions of the dihydronicotinamides **12–15** catalyzed by 8-acetyl-10-methylisoalloxazine (**8**) under aerobic conditions and with the dihydronicotinamide in excess were shown to be pseudo-first-order processes, at least through the first 95% of the reaction. The pseudo-first-order rate constants, $k_{obsd}$, were found to be directly proportional to the flavin concentration, and the second-order rate constants calculated for a series of 1-alkyldihydronicotinamides are presented in Table I.

These kinetic results, consistent with first-order dependences of the oxidation rate law on the dihydronicotinamide and flavin concentration, were obtained in the presence of a quantity of the enzymes superoxide dismutase and catalase in the reaction mixture. The function of these enzymes is envisioned to be the removal of the reduced oxygen species $O_2^{-}\cdot$ and $H_2O_2$ produced by oxidation of the product dihydroflavin by oxygen. It is well-known that dihydronicotinamides can react by free radical mechanisms initiated by superoxide anion, and that superoxide can arise as a byproduct of the reaction of the dihydroflavin with oxygen.[27] Removal of the superoxide anion radical is expected to prevent these dihydronicotinamide-consuming free radical processes. As anticipated, inclusion of the scavenger enzymes in the reaction mixture clearly changed the kinetics and resulted in a diminished rate for the oxidation of the dihydronicotinamide substrate.

The kinetics of the oxidation of 1-propyl-1,4-dihydronicotinamide (**12**) catalyzed by the related 6-acetyl-10-methylisoalloxazine (**11**) was also examined and found to present additional complications. The kinetics studied under aerobic conditions, with dihydronicotinamide in excess, and in the absence of scavenger enzymes was clearly not a first-order process with respect to flavin.

(21) Singer, T. P.; Kearney, E. B. *J. Biol. Chem.* **1950**, *183*, 409–429.
(22) Bruice, T. C.; Main, L.; Smith, S.; Bruice, P. Y. *J. Am. Chem. Soc.* **1971**, *93*, 7327–7328.
(23) Porter, D. J. T.; Blankenhorn, G.; Ingraham, L. L. *Biochem. Biophys. Res. Commun.* **1973**, *52*, 447–452.
(24) Blankenhorn, G. *Eur. J. Biochem.* **1975**, *14*, 3172–3176.
(25) This program for the estimation of parameters by nonlinear regression was obtained from B. A. Blumenstein, Emory University.
(26) Initial rates were fit to a hyperbolic $V$ vs. [$S$] plot by using an iterative curve fitting 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.
(27) Porter, D. J. T.; Bright, H. J. *J. Biol. Chem.* **1980**, *255*, 7362–7370.

In fact, the rate constant $k_{obsd}$ depended in a nonlinear fashion upon the flavin concentration. When **11** was used as a catalyst for the oxidation of 1-propyl-1,4-dihydronicotinamide (**12**), the following rate equation was established:

$$v = 23[6\text{AcFl}]^{0.8}[\text{NPrNH}]$$

The kinetics of the oxidation of 1-benzyl-1,4-dihydronicotinamide (**14**) by isoalloxazine **11** obeyed the same type of rate equation. It is therefore reasonable to conclude again that the process cannot be regarded as a true second-order reaction, but rather as one in which a free radical initiated process makes a significant contribution. Employment of scavenger enzymes to prevent these reactions in this case caused the kinetics to deviate from a strict pseudo-first-order process. It was therefore necessary to use initial rate measurements to determine the second-order rate constant for the oxidation of 1-benzyl-1,4-dihydronicotinamide (**14**) by isoalloxazine **11**. The double-reciprocal plot was linear, and the value of the second-order rate constant so defined is presented in Table I. From our experimental data it can be concluded that if preequilibrium complex formation occurs in this reaction, the dissociation constant for the complex must be greater than $10^{-3}$ M.

**Oxidation of Dihydronicotinamides Catalyzed by Flavopapain 6 and 9.** Flavopapain **6** was found to catalyze the oxidation of NADH and related 1-alkyl-1,4-dihydronicotinamides **12–14**. Under aerobic conditions with the concentration of the nicotinamide in excess, saturation kinetics were observed. That is, the kinetics of the oxidation are described by eq 1.

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

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

$$\text{EH}_{2(red)} + \text{O}_2 \xrightarrow{k_o} E_{(ox)} \quad (2b)$$

The values of the kinetic constants can be extracted from the total time course of the enzymatic reaction using an iterative curve-fitting computer program.[25] Alternately, they can be derived by the method of initial rates, using either the graphical procedure of Lineweaver and Burk or computer curve-fitting programs to determine exact values of $k_{cat}$ and $K_m$.[26] (See Table II.)

Kinetic constants derived from the analysis of the total time course for the oxidation and those determined using the method of initial rates were found to agree only when scavengers capable of removing the superoxide anion (i.e., superoxide dismutase) and hydrogen peroxide (i.e., catalase) were present in the reaction mixture. The ability of superoxide anion, produced by the oxidation of the dihydroflavin by $O_2$, to initiate free radical chain oxidations of 1-propyldihydronicotinamide has already been reported.[27] These chain reactions were found to account for a significant amount of the oxygen consumption (and therefore the consumption of reduced nicotinamide) during the aerobic oxidation of 1-propyldihydronicotinamide by lumiflavin. This chain reaction could be eliminated by removal of $O_2^{-}\cdot$ from the solution by including either $Cu^{2+}$ salts or superoxide dismutase in the reaction mixture. Interestingly, NADH and NADPH were noted to react poorly with $O_2^{-}\cdot$, and free radical chain oxidations did not occur using these substrates even in the absence of a superoxide trap.

When the rate parameters $k_{cat}/K_m$ for the oxidation of dihydronicotinamides **12–15** by 8-acetylflavopapain (**6**) are compared to the second-order rate constants for the oxidation reactions catalyzed by the simple model flavin **8**, it is immediately evident that high catalytic rate enhancements are achieved by the flavopapain. The best flavopapain substrate, 1-hexyl-1,4-dihydronicotinamide (**13**) is oxidized 620 times faster by the semisynthetic enzyme **6** than it is by the 8-acetyl flavin **8**. This is a significant catalytic rate enhancement, considerably exceeding the 20-fold rate enhancement achieved by our next most successful semisynthetic enzyme, flavopapain **2**. The oxidation of 1-benzyl-1,4-dihydronicotinamide (**14**) by flavopapain **6** shows the next largest catalytic rate enhancement relative to the corresponding model reaction, ca. 200-fold. It is significant that our model enzyme **6** is not only efficient in its oxidation of substrates but is also increasingly selective: 1-propyl-1,4-dihydronicotinamide is oxidized at one-tenth the rate of 1-hexyl-1,4-dihydronicotinamide, and the poorest substrate, NADH, exhibits a very modest 4-fold catalytic rate enhancement. This is in accord with our expectation that binding of the 1-alkyl chains of our substrates are being affected by the *hydrophobic* binding grove of papain, thus favoring the interaction of the hexyl side chain over the propyl and favoring all of the 1-alkyldihydronicotinamides over the hydrophilic and bulky NADH.

**Table II.** Rate Constants for the Oxidation of Dihydronicotinamides by Flavopapains **6** and **9**[a]

| dihydronicotinamide | $K_m$, μM | $k_{cat}$, $s^{-1}$ | $k_{cat}/K_m$, $M^{-1}$ $s^{-1}$ |
|---|---|---|---|
| | Flavopapain **6** | | |
| NBzNH **14** | 2.7 ± 0.3 | 0.093 ± 0.009 | 33 800 |
| (by initial rate) | 2.7 ± 0.5 | 0.090 ± 0.007 | 32 900 |
| NPrNH **12** | 0.812 ± 0.8 | 0.048 ± 0.003 | 58 700 |
| (by initial rates) | 0.765 ± 0.95 | 0.047 ± 0.0008 | 61 300 |
| NHxNH,[b] **13** | 0.118 ± 0.1 | 0.067 ± 0.0009 | 570 000 |
| NADH,[c] **15** | 349 ± 30 | 0.0073 ± 0.0005 | 21 |
| | Flavopapain **9** | | |
| N-BzNH, **14** | 456 | 0.036 | 78 |

[a] Rates measured at 25 °C in 0.1 M Tris·HCl, 0.1 mM EDTA, pH 7.5, with 0–1% ethanol. Each solution of 3.0 mL contains 10 units of superoxide dismutase and 3500 units of catalase. [b] Rates measured in a 5-cm length cell at an ambient temperature of 27 °C. Other conditions identical. [c] Rates measured in the absence of superoxide dismutase and catalase.

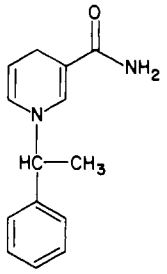
Although the dissociation constants for enzyme–substrate complex formation are expected to be reflected in the magnitude of $K_m$, it is our expectation that $K_m$ and $k_{cat}$ will prove to be kinetically complex for the oxidation of dihydronicotinamides by flavopapain **6**. Therefore, $K_m$ should not be interpreted as a measure of the actual thermodynamic substrate binding constant. Our expectation of complex kinetics is based partly on the observation that oxidation of benzyldihydronicotinamide **14** by the flavopapain **2** studied under anaerobic conditions by fast reaction techniques has been demonstrated to involve the transient formation of an intermediate which follows the enzyme–substrate complex but precedes the hydride transfer. Secondly, preliminary rapid kinetic studies of the oxidation of benzyldihydronicotinamide by the flavopapain **6** indicates that under the conditions used in our experiments the reoxidation of the dihydroflavin by oxygen (eq 2b) may affect significantly the rate of the enzymatic oxidation.[28] Additional intermediates may be present along the oxidation pathway as well.

Studies of the kinetics using 1-benzyl-1,4-dihydronicotinamide (**14**) showed that attachment of the isoalloxazine molecule to the papain active site via the 6α-acetyl linkage does not create a more efficient catalyst than the model system itself. The observed $k_{cat}/K_m$ is essentially the same as $k_2$ for the model flavin (see Tables I and II). These results indicate the very critical role played by positioning of the isoalloxazine at the active site. The hydrogen bonding of the 6α-acetyl carbonyl with the protein backbone is envisioned in this case to require the flavin to reside in a position remote from the substrate binding groove and thus renders the model enzyme ineffective.[29]

(28) Although $K_m$ appears to be complex and the rate of oxidation of the dihydroflavin species by oxygen may be rate-limiting under certain circumstances, a concentration term in oxygen will not appear in the expression for $k_{cat}/K_m$ unless the mechanism requires a ternary complex of flavin, dihydronicotinamide, and oxygen to occur in a kinetically significant step. Measurements of the flavopapain **6** catalyzed oxidiation of dihydronicotinamides in the presence of dichloroindophenol which reacts more rapidly with the dihydroflavin than does oxygen give $k_{cat}/K_m$ values in good agreement with those measured in the presence of oxygen. Therefore, we do not believe that the $k_{cat}/K_m$ values reported in this manuscript are significantly affected by oxygen concentration.

**Stereospecificity of Oxidations by 8-Acetylflavopapain (6).** It is well-known that enzymic catalysts are not only highly efficient but that they are capable of exhibiting high stereoselectivity as well. Indeed, this is one of the principal motivations behind biomimetic chemistry—the desire to develop highly selective catalysts for asymmetric synthesis. Hence, early in the course of our studies on flavopapain **6**, we asked what the effects of the existence of an asymmetric carbon in the side chains of our substrates would be. In order to do this, it was necessary to modify the synthesis of the 1-alkylnicotinamides. Although high yields of 1-alkylnicotinamide salts can be obtained directly by the alkylation of nicotinamide with primary halides (benzyl chloride, ethyl and propyl iodide, etc.), this is not the case with secondary halides. Attempts to alkylate nicotinamide with $\alpha$-phenethyl bromide, for example, lead only to the isolation of the hydrobromide salt of nicotinamide. Apparently, elimination can compete favorably with alkylation. Fortunately, another synthesis of alkylnicotinamides was available (Scheme II) based upon the Zinke Reaction.[28] Here, primary amines are observed to react with 1-(2,4-dinitrophenyl)-3-carbamoylpyridinium salts to give the 1-alkylnicotinamide derived from the amine and 2,4-dinitroaniline as products. Since the reaction does not involve breaking of the C–N bond in the primary amine, optically active amines will produce optically active nicotinamides, with the identical absolute configuration.[29]

In order to detect small differences in the ability of enantiomers to serve as substrates, a set of chiral dihydronicotinamides was made by reacting either *d*-(+)- or *l*-(−)-$\alpha$-methylbenzylamine with 1-(2,4-dinitrophenyl)-3-carbamoylpyridinium tetrafluoroborate, followed by isolation of the amorphous nicotinamide salt and reduction to the dihydronicotinamide **23** and **24**, isolated as yellow oils.



**23**, D enantiomer
**24**, L enantiomer

Both of these derivatives were good substrates for 8-flavopapain **6**, exhibiting kinetic behavior comparable to that of 1-benzyl-1,4-dihydronicotinamide. The kinetics observed for the oxidation of both enantiomers was identical to within experimental error. Flavopapain **8**, thus fails to discriminate between enantiomers where the asymmetric carbon is attached to the 1-pyridine nitrogen.

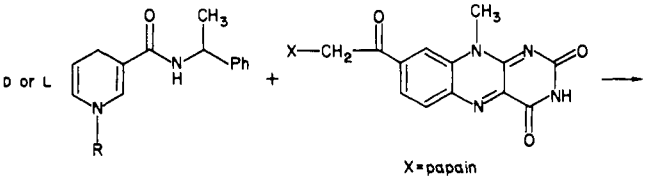
We should not be too disappointed with this result since model building would indicate that these chiral nicotinamide derivatives can be positioned so that the pyridine ring is adjacent to the flavin moiety, and the alkyl side chain is extended into the hydrophobic binding site in such a way that the asymmetric carbon atom does not make any close contacts with the residues on the protein surface. Hence, both enantiomers are effective substrates.

(29) In view of the complex kinetics observed we must be satisfied with an estimate for the aerobic rates of oxidation of dihydronicotinamide **14** catalyzed by the model flavin **11** or by the corresponding flavopapain **9**. Although we have not determined the reasons for this complexity, our rate measurements were carried out using roughly equivalent concentrations of catalysts **9** and **11** under otherwise identical experimental conditions. We conclude, therefore, that the rates of oxidation achieved by both of the catalysts are approximately equivalent.

(30) Zincke, Th.; *Ann.* **1903**, *330*, 361–374. Zincke, Th.; Heuser, G.; Möller, W. *Ann.* **1904**, *333*, 296–345. Zincke, Th.; *Ann.* (**1905**,) *341*, 369–379.

(31) de Gee, A. J.; Sep, W. J.; Verhoeven, J. W.; de Boer, T. J. *J. Chem. Soc. Perkin Trans. 1* **1974**, 676–679.

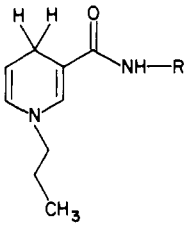
**Table III.** Specificity of Flavopapain for Optically Active Substrates



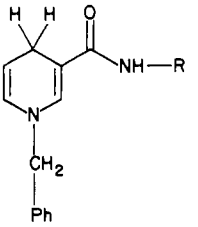
X = papain

| dihydronicotinamide | $K_m$, μM | $k_{cat}$, $s^{-1}$ | $k_{cat}/K_m$, $M^{-1}$ $s^{-1}$ | ratio of rate constants L/D |
|---|---|---|---|---|
| L-(+), R = *n*-$C_3H_7$ | 6.0 | 0.07 | 12000 | 2.4 |
| D-(−), R = *n*-$C_3H_7$ | 19.0 | 0.09 | 5100 | |
| L, R = $CH_2Ph$ | 22 | 0.08 | 3800 | 1.7 |
| D, R = $CH_2Ph$ | 31 | 0.08 | 2200 | |

A more suitable set of derivatives with which to find a chiral discrimination are those substituted dihydronicotinamides described by Ohno and co-workers[18] in which the nitrogen of optically active $\alpha$-methylbenzylamide is substituted for the $NH_2$ in the carboxamide side chain.



**25**, R = D-$CHCH_3Ph$
**26**, R = L-$CHCH_3Ph$



**27**, R = D-$CH(CH_3)Ph$
**28**, R = L-$CH(CH_3)Ph$

These compounds were found to be good substrates for the flavopapain **6** as well, albeit exhibiting somewhat (i.e., 10-fold) higher values for $K_m$. The kinetic data for these two sets of derivatives are presented in Table III. It is apparent that chiral discrimination between the D and L enantiomer occurs, with the L isomer favored in each case by a factor of approximately 2.

This readily detectable chiral discrimination (2-fold) is certainly sufficient to establish that the active site of flavopapain **6** is asymmetric, as is required of an enzymelike active site. It is not, however, great enough for meaningful asymmetric synthesis or for kinetic resolution of a racemate. An objective of future work in this area must be the design of substrates, based upon a conception of the flavopapain active site and model building, capable of exhibiting preferential binding and reaction of a single enantiomer out of the pair.

In summary, we have successfully modified papain with a series of isoalloxazines which are related as geometric isomers. Despite the similar chemical reactivity of the three parent isoalloxazines, the resultant flavopapains show widely different rate enhancements for catalysis of the oxidation of 1-benzyl-1,4-dihydronicotinamide by oxygen. The observed catalytic rate enhancement is essentially absent for flavopapain **9**, is about 1 to two orders of magnitude for flavopapain **2**, and is two to almost 3 orders of magnitude for the most effective flavopapain **6**. The differences in the efficacy of our flavopapains are best explained in terms of differing geometries in the active sites of the semisynthetic enzymes. The activity of the semisynthetic enzymes within this series lends support to our working hypothesis that proper positioning of the flavin by the hydrogen binding of the acetyl group to residues in the protein backbone is crucial in the production of an effective semisynthetic active site. In this respect our semisynthetic active site resembles its natural counterparts where subtle geometric differences in the active site contribute greatly to specificity.

**Acknowledgment.** This work was supported by NSF Grant CHE 8218637 (E.T.K.) and USPHS Postdoctoral Fellowship GM-07766-03 (J.T.S.).