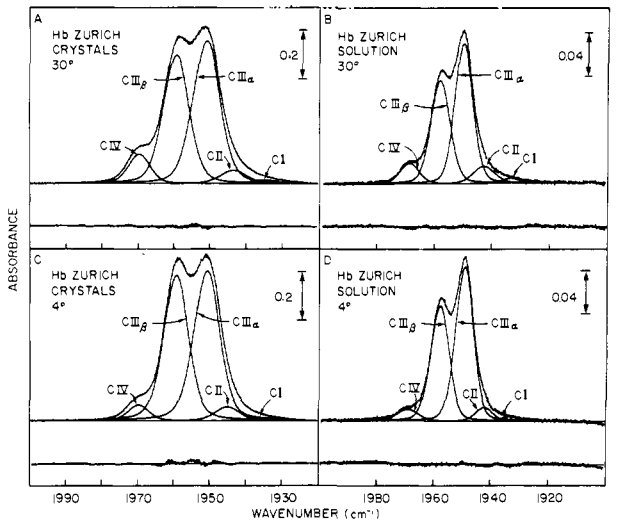

Figure 2. Infrared spectra of HbZhCO in crystals and in solution: (A) crystals at 30 °C, (B) solution at 30 °C, (C) crystals at 4 °C, (D) solution at 4 °C. Conditions and spectral methods are equivalent to those described for HbA in Figure 1. The residual difference between the observed spectrum and the sum of the bands of deconvolution is shown offset below the base line for each spectrum.

band near 1951 cm^{-1} under normal solution conditions, the human mutant Hb Sydney CO (β67 Val→Ala) exhibits a β CIII band at 1955 cm^{-1} and an intensified CIV band at 1968 cm^{-1}.[10] Thus, replacement of Val by the less bulky Ala at Ell, a residue in van der Waal's contact with the CO ligand, results in changes in solution spectra not unlike those found for HbACO upon crystallization.

The β E7 distal His to Arg substitution in HbZhCO (Figure 2) also results in marked changes in the C-O stretch bands, i.e., in solution the β CIII band is at 7-cm^{-1} higher frequency and band CIV is enhanced. The spectra for the normal α subunits of HbZhCO are similar to α HbACO spectra. In solution, the α CIII band of HbZhCO is more intense than the β CIII band. Upon crystallization, the two CIII bands become closer in intensity (Table I). Decreasing the temperature of either crystals or solutions from 30 to 4 °C shifts intensity from minor conformers (CI, CII, CIV) to the major conformer (CIII); thus, interconversion among conformers occurs in crystals as well as solutions. With HbZhCO, crystallization has little effect upon ν_{CO} values. Therefore ligand binding site structures remain essentially unchanged.

In summary, C–O stretch bands provide direct evidence of differences and similarities between crystalline and solution states in terms of conformer structures and relative conformer stabilities. The ν_{CO} values reflect a marked change in ligand binding site structure for the β subunit of HbACO upon crystallization but very little change in the α subunit. With HbZhCO, crystal formation has a smaller effect upon conformer structures. With both hemoglobins, crystallization alters the relative stability of conformers. These infrared results provide evidence that discrete, interconvertible conformers of roughly comparable structures are present in both crystals and solutions. It is also clear that ligand infrared spectra can be uniquely useful for the comparison of crystal and solution protein structures at ligand binding sites.

Acknowledgment. This work was supported by NIH Grant HL 15980. The kindness of Dr. Samuel Charache for providing samples of blood from patients that contained HbZh is greatly appreciated.

Registry No. HbACO, 9072-24-6; HbZhCO, 9072-31-5.

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Catalysis of *N*-Alkyl-1,4-dihydronicotinamide Oxidation by a Flavopapain: Rapid Reaction in All Catalytic Steps

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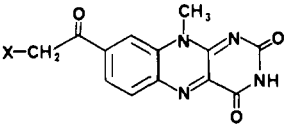
In the course of research on the design and construction of new enzymes we have succeeded in linking various flavins to papain, converting this hydrolytic enzyme into semisynthetic enzymes capable of catalyzing the oxidation of *N*-alkyl-1,4-dihydronicotinamides with high efficiency.[1-5] The most effective of the flavopapain catalysts prepared to date has been the semisynthetic enzyme obtained by the alkylation of the active site Cys-25 residue of papain with 8α-(bromoacetyl)-10-methylisoalloxazine.[4,5] The k_{cat}/K_m value measured at 25 °C and pH 7.5 under aerobic conditions for the oxidation of *N*-hexyl-1,4-dihydronicotinamide by various electron acceptors as catalyzed by this flavopapain **I**



1; X=papain–S

2; X=H

is in the vicinity of 10^6 M^{-1} s^{-1}. This constitutes an approximately 10^3-fold rate increase when compared to the second-order rate constant for the corresponding oxidation reaction catalyzed by the model compound **2**. To understand better the origin of the efficiency of this flavopapain it is important to dissect the observed rate parameters measured under turnover conditions into the microscopic rate parameters for the reaction. Through elucidation of the way in which this rate acceleration arises, it may be possible to learn how to optimize the design of other semisynthetic enzymes. Furthermore, the placement of cofactors into known three-dimensional environments permits the systematic investigation of the factors important for efficient catalysis.

We have used anaerobic conditions and stopped-flow techniques[6] to investigate the individual steps in the oxidation of *N*-alkyl-1,4-dihydronicotinamides catalyzed by flavopapain. We found that at 25.0 °C, under conditions of substrate in excess, the reduction of flavopapain by the *N*-alkyl-1,4-dihydronicotinamides

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Table I. Rate Parameters for Oxidation Reactions Catalyzed by **1** and **2**

	electron acceptor	flavopapain **1** k_{cat}, s^{-1}	flavopapain **1** K_m, μM	flavopapain **1** k_{cat}/K_m, M^{-1} s^{-1}	flavin **2** k_2, M^{-1} s^{-1}	flavopapain **1** red.[d] k_2, M^{-1} s^{-1}	flavin **2** red.[d] k_2, M^{-1} s^{-1}
NHexNH	none[a]	370 ± 70	230 ± 50	$1.64 \times 10^6 \pm 0.03 \times 10^6$			
	O_2[b]	0.067	0.12	5.7×10^6	917 ± 18		
	MTT				1345 ± 35		
NBzNH	none[a]	29 ± 5.5	471 ± 91	$61.8 \times 10^3 \pm 21 \times 10^3$			
	O_2[c]	0.13 ± 001	2 ± 0.3	$67.4 \times 10^3 \pm 10 \times 10^3$	170 ± 2		
	MTT	31.5 ± 1.0	540 ± 17	$58.8 \times 10^3 \pm 0.5 \times 10^3$	210 ± 10		
NPrNH	none[a]	31 ± 11	210 ± 79	$1.79 \times 10^5 \pm 0.22 \times 10^5$			
	O_2[b]	0.056	0.65	86×10^3	878 ± 23		
	MTT	62.5 ± 9.5	1000 ± 160	$60.9 \times 10^3 \pm 1 \times 10^3$	1150 ± 10		
dichloroindophenol						1.5×10^7	9×10^7
coenzyme Q_0						7.1×10^5	7×10^6
cytochrome C						4.3×10^5	

[a] Refers to anaerobic conditions. Measured at 24.5 °C in 67 mM HEPES pH 7.6 with stopped-flow spectrophotometer. [b] Measured at 25 °C in 0.1 mM Tris·HCl, 0.1 mM EDTA, pH 7.5, air-saturated buffer. Each solution of 3-mL volume contained 10 units of superoxide dismutase and 3500 units of catalase. [c] Enzymatic rates were measured in oxygen-saturated Tris·HCl, 0.1 mM EDTA pH 7.5 buffer containing the above-mentioned scavenging enzymes. [d] Obtained by photochemical reduction with EDTA in the presence of catalytic amounts of 5-deazariboflavin. Measured under anaerobic conditions in stopped-flow spectrophotometer.

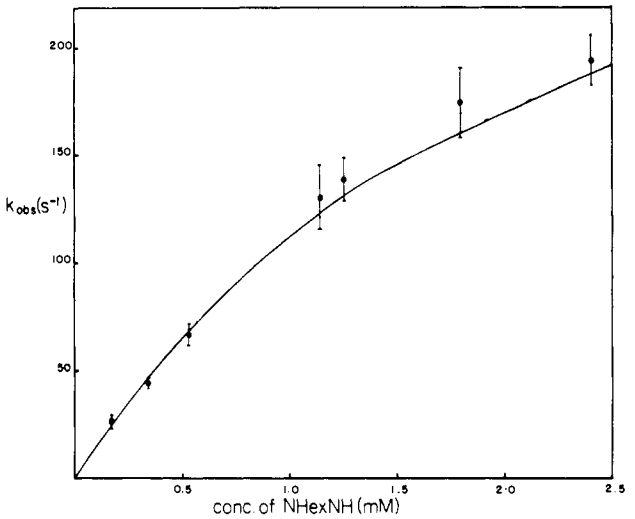

Figure 1. Concentration dependence of observed pseudo-first-order rate constant for the reduction of 7.6 μM flavopapain **1** by *N*-hexyl-1,4-dihydronicotinamide at pH 7.5 and 24.5 °C.

exhibited pseudo-first-order kinetics as observed at 450 and 530 nm.[7] Plots of the observed first-order rate constant vs. the substrate concentration indicated that saturation conditions could be approached for the three dihydronicotinamides studied (Figure 1). The anaerobic data for the reactions of the dihydronicotinamides with flavopapain **1** are summarized in Table I[8] and are consistent with the scheme of eq 1. The corresponding reactions of the benzyl-, propyl- and hexyl-1,4-dihydronicotinamides with the model flavin **2** were also measured anaerobically (Table I). In contrast to the behavior of flavopapain **1**, the model reactions showed no evidence for saturation up to concentrations of the dihydronicotinamides of 400 μM. Comparison of the second-order rate constants for the anaerobic reduction of model compound **2** by the *N*-alkyl-1,4-dihydronicotinamides with the corresponding parameter k_2/K_s for the enzymatic reactions shows that the enzymatic reactions proceed from 10^2 to 10^3 times faster than the model processes. It is clear also from Table I that the enzymatic reactions proceed rapidly in large part because the value of k_2, the rate constant for the conversion of the Michaelis complex to the reduced dihydroflavoenzyme, is very large. Indeed, for the *N*-hexyl compound the value is so large that even with stopped-flow techniques only an estimate of the rate constant can be obtained. The rapidity with which this important catalytic step takes place is reminiscent of what has been observed with the most effective naturally occurring flavoenzymes:

$$E_{ox} + NRNH \underset{K_s}{\rightleftharpoons} E_{ox}\cdot NRNH \xrightarrow{k_2} EH_2 + NRN \quad (1)$$

where E_{ox} is the oxidized form of flavopapain, EH_2 is the reduced form of flavopapain, E_{ox}·NRNH is the Michaelis complex, NRNH is dihydronicotinamide, and NRN is nicotinamide.

It was also possible to examine the reaction of reduced flavopapain with various acceptors. As can be seen from Table I, oxygen is a poor acceptor under steady-state conditions. Its reaction with dihydroflavopapain is similar to what is observed with model dihydroflavins,[9,10] showing autocatalytic behavior and producing superoxide, which itself carries out further oxidation reactions. The addition of 1 μM superoxide dismutase suppresses the overall reaction rate by about a factor of 2. It is interesting to note that the reaction of the dihydro form of flavoenzyme **1** with oxygen is about 7 to 8 times slower than the corresponding reaction seen with the dihydro form of model flavin **2**.

Dichloroindophenol, coenzyme Q_0, and cytochrome C all act as excellent electron acceptors from the dihydro form of either the model flavin or flavopapain. As in the case of the oxygen reaction the reduced form of the enzyme reacts appreciably more slowly, however, with dichloroindophenol and with coenzyme Q_0 than does the model reduced compound. Although cytochrome C is undoubtedly reduced by flavopapain in one-electron steps, no evidence for a semiquinone form of the enzyme has been observed in the electron-transfer step.

In Table I are shown the data obtained under turnover conditions when the oxidation of the dihydronicotinamides by flavopapain is carried out in the presence of either air- or oxygen-saturated buffer. By comparison of the data for reduction of the flavopapain under anaerobic conditions and for the reaction of oxygen with the dihydroflavin, it is clear that under the turnover conditions used for the aerobic reactions the reaction of oxygen with the dihydroflavin form of flavopapain is largely rate limiting in the substrate concentration range that we employed. In order to achieve turnover conditions where the reduction step rather than the oxidation of the dihydroflavin is rate limiting we must use an acceptor that reacts more quickly than oxygen. One example of such an acceptor is 3-(4,5-dimethylthiazol-2-yl)-2,4-diphenyltetrazolium bromide (MTT). As can be seen from the data measured for MTT and shown in Table I, by the use of concentrations of MTT in the vicinity of 400 μM it is possible to measure k_{cat} values for the oxidation of *N*-benzyl- and *N*-propyl-1,4-dihydronicotinamides which correspond closely with those obtained

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under anaerobic conditions for the reduction of the flavopapain by these substrates.

In summary, using *N*-alkyl-1,4-dihydronicotinamides as reductants and a variety of appropriate electron acceptors we have shown that a flavoenzyme exhibiting high catalytic efficiency both in terms of k_{cat}/K_m and of the value of the turnover number can be constructed by utilizing the active and binding sites of the hydrolytic enzyme papain.

Acknowledgment. Support of this research by National Science Foundation Grant CHE82-18637 (E.T.K.) and National Institutes of Health Grant GM20877 (D.P.B.) is gratefully acknowledged.

Trichothecene Metabolism Studies: Isolation and Structure Determination of 15-Acetyl-3α-(1′β-D-glucopyranosiduronyl)-scirpen-3,4β,15-triol

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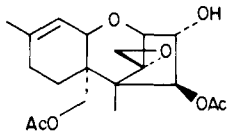
The epoxytrichothecene mycotoxins are a group of fungal metabolites that exhibit a range of significant biological properties including cyto- and phytotoxicity.[3] These mycotoxins are potent inhibitors of protein synthesis in eukaryotes and have been implicated in a number of diseases of plants, animals, and humans.[3a] Certain members of this group, including anguidine (**1**) and T-2 toxin (**2**), have also received considerable notoriety as a consequence of the "yellow rain" controversy.[4]



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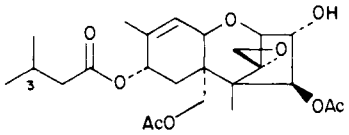
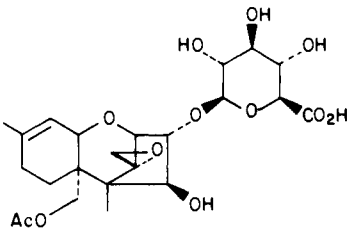
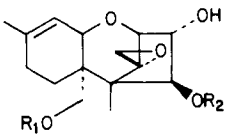


2

In spite of the toxicological significance of the epoxytrichothecenes, relatively little is known about their metabolic fate in mammalian systems.[3,5] Anguidine, for example, has been examined in dogs and monkeys mainly for toxic manifestations.[6,7] The only metabolic transformations documented prior to the initiation of our studies had been deacylation reactions of the trichothecene esters mediated by microsomal esterases,[5c,8] the cytochrome P_{450} catalyzed oxidations of T-2 and HT-2 toxin leading to 3′-hydroxyl derivatives,[5a,b] and the deoxygenation of the 12,13-epoxide of deoxynivalenol.[5d] We report herein the isolation and structure determination of glucuronide **3**, the major metabolite of anguidine in *in vitro* studies, and also present evidence that glucuronidation occurs in vivo.[9] This is the first rigorous demonstration that glucuronidation is a significant pathway for trichothecene metabolism.



3



4. R_1=Ac, R_2=H
5. R_1=H, R_2=Ac

[^{3}H]Anguidine (12 μM, 0.16 μCi)[10] was incubated with uridine 5′-diphosphoglucuronic acid (UDPGA, 12 mM), β-naphthoflavone-induced hepatic microsomes from male Long-Evans rats (0.6 mg of protein/mL),[11] $MgCl_2$ (2.5 mM), and K_2HPO_4 (10 mM, pH 7.7) at 37 °C. HPLC analysis of the mixture after 1.5 h indicated that three new products were present: glucuronide **3** (38–50%; R_t = 12.5 min), 15-acetoxyscirpendiol (**4**) (43–32%; R_t = 22 min), and 4-acetoxyscirpendiol (**5**) (18–19%; R_t = 18 min).[12-14] After a 3.3-h incubation, the mixture consisted of **3** (68–73%), **4** (1–4%), and **5** (22–23%).[15] Scaleup of this procedure[16] (12 mg of anguidine) afforded 9 mg (56%) of **3**[17] following HPLC purification.

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(2) Holder of the Roger and Georges Firmenich Career Development Chair in Natural Products Chemistry, 1981–1984; Fellow of the Alfred P. Sloan Foundation, 1982–1984.

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(9) A complete account of our investigation of the metabolism of anguidine will be reported separately: Marletta, M. A.; Roush, W. R.; Recchia, J.; Russo-Rodriguez, S., manuscript in preparation. We have looked for, but failed to find any evidence of, metabolic products stemming from cytochrome P_{450} oxidation, epoxide hydrolysis, or glutathione conjugation.

(10) [^{3}H]Anguidine (13.4 mCi/mmol) was prepared by Dr. T. J. Caggiano by NaB^3H_4 reduction of 4β,15-diacetoxyscirpen-3-one. We thank Dr. Doyle of Bristol Laboratories for providing a copy of this procedure prior to publication.

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(12) The microsomes were removed by centrifugation at the end of the incubation. The metabolites were concentrated on a C18 SEP-PAK cartridge (Waters Assoc.) that had been preequilibrated in water, eluted with MeOH, and then separated by HPLC (see ref 13).

(13) A μBondapak C18 column (3.9 mm × 30 cm; Waters Assoc.) was used for all analyses and isolations (100% H_2O for 2 min, 0–45% MeOH linear ramp for 13 min, and a 45–60% MeOH linear ramp for 15 min; 1.5 mL/min).

(14) Metabolites **4** and **5** were identified by GC/MS analysis with comparison to authentic samples. We thank Dr. J. S. Wishnok for assistance with these analyses.

(15) These data suggest that anguidine is rapidly hydrolyzed to a mixture of monoacetates **4** and **5** and that glucuronidation then occurs with **4** in a subsequent step. In support of this hypothesis we have found that incubation of [^{3}H]-**4** with liver microsomes and UDPGA, or **4** with [^{14}C]-UDPGA, according to the procedure described in the text also afforded glucuronide **3**.

(16) UDPGA (12 mM), anguidine (645 μM), and 1.16 mg/mL (29 mg total) of microsomal protein were incubated for 3.3 h.

(17) Data for **3**: $[\alpha]^{22}_D$ − 44.7° (*c* 0.44, 99.8% MeOH); ^{1}H NMR (MeOH-d_4, 250 MHz) δ 5.44 (br d, 1 H, *J* = 5.2 Hz, H-10), 4.67 (d, 1 H, *J* = 7.8 Hz, H-1′), 4.43 (m, 2 H, H-3 and H-4), 4.20 and 3.88 (AB, 2 H, *J* = 12.3 Hz, H-15), 4.00 (d, 1 H, *J* = 5.4 Hz, H-11), 3.67 (d, 1 H, *J* = 4.5 Hz, H-2), 3.61 (m, 1 H, sugar), 3.45 (m, 2 H, sugar), 2.95 and 2.77 (AB, 2 H, *J* = 4.1 Hz, H-13), 2.04 (s, 3 H, -OAc), 1.70 (s, 3 H, H-16), 0.80 (s, 3 H, H-14); FT-IR (solid film on CaF_2 window) 3500–3200 (br, OH), 2966, 2910, 1739, 1653, 1616, 1613, 1419, 1415, 1387, 1374, 1366, 1242, 1159 cm^{-1}; mass spectrum (FAB, glycerol dispersion), *m/e* 501 (MH^+), 307, 265.

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