ters are the mass of the black hole and the angular momentum of the black hole. What is even more remarkable, the metric describing these solutions is simple and can be explicitly written down.

I do not know if the full import of what I have said is clear. Let me explain.

Black holes are macroscopic objects with masses varying from a few solar masses to millions of solar masses. To the extent they may be considered as stationary and isolated, to that extent, they are all, every single one of them, described *exactly* by the Kerr solution. This is the only instance we have of an exact description of a macroscopic object. Macroscopic objects, as we see them all around us, are governed by a variety of forces, derived from a variety of approximations to a variety of physical theories. In contrast, the only elements in the construction of black holes are our basic concepts of space and time. They are, thus, almost by definition, the most perfect macroscopic objects there are in the universe. And since the general theory of relativity provides a single unique two-parameter family of solutions for their descriptions, they are the simplest objects as well.

Turning to the physical properties of the black holes, we can study them best by examining their reaction to external perturbations such as the incidence of waves of different sorts. Such studies reveal an analytic richness of the Kerr space-time which one could hardly have expected. This is not the occasion to elaborate on these technical matters (*22*). Let it suffice to say that contrary to every prior expectation, all the standard equations of mathematical physics can be solved exactly in the Kerr space-time. And the solutions predict a variety and range of physical phenomena which black holes must exhibit in their interaction with the world outside.

The mathematical theory of black holes is a subject of immense complexity. But its study has convinced me of the basic truth of the ancient mottoes, "The simple is the seal of the true" and "Beauty is the splendour of truth."

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Chemical Mutation of Enzyme Active Sites

E. T. Kaiser and D. S. Lawrence

A major objective of biochemists continues to be the elucidation of the structural basis of enzyme function and activity. The understanding of the relation between enzyme structure and activity has been facilitated by x-ray crystallographic studies, chemical modification experiments, kinetic investigations, and other related techniques. These kinds of experimentation have yielded considerable information concerning two principal phenomena that underlie the activity of enzymes: substrate binding and the subsequent intracomplex catalysis.

The information on structures now available on enzymes, and knowledge of the pathways by which enzymatic catalysis occurs, have made it possible for the chemist to contemplate designing enzymes from their constituent amino acids and cofactors. The clear prediction of the pattern of the folding of long peptide chains into specific tertiary structures still poses many difficulties. Therefore, the construction of new enzymes through total synthesis still remains a goal. Nevertheless, a breakthrough has been made in a major aspect of the problem of enzyme design, the creation of new enzyme active sites, by a process that we term "chemical mutation" (*1–7*).

In the chemical mutation approach, the starting material is a natural protein that has folded to a stable conformation. Groups at or on the periphery of the active site are chemically modified to produce a "semisynthetic" enzyme having catalytic activity different from that of the original enzyme. An attractive feature of the chemical mutation process is that the wealth of x-ray structural information available for relatively simple enzymes makes possible a considerable degree of flexibility in the choice of the natural system in which the new catalytic group is introduced. Until now, much of the effort has been on the conversion of readily available enzymes of moderate molecular weight that are hydrolytic catalysts into modified enzymes capable of catalyzing other important reactions such as oxidation-reduction, decarboxylation, and transamination. We have shown that we can achieve the "chemical mutation" of enzyme active sites by the reaction of appropriate coenzyme analogs containing reactive functional groups with amino acid residues in or near active sites of hydrolytic enzymes. With suitably chosen enzyme templates, appropriate coenzyme ana-

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logs can be covalently attached in a manner permitting the enzyme binding sites to remain accessible to organic substrates as shown in Eq. 1.

Active site residue
+
Reactive coenzyme analog
↓
Semisynthetic enzyme containing covalently bound coenzyme (1)

The choice of an appropriate enzyme as the starting material for the preparation of a semisynthetic enzyme has been made with consideration to five criteria.

1) The enzyme to be used should be readily available in highly purified form.

2) The x-ray structure of the enzyme should be known.

3) The enzyme should have a suitably reactive amino acid functional group at or near the active site.

4) The covalent modification of the enzyme should result in a significant change in the activity characteristic of the native enzyme.

5) The attachment of the coenzyme analog should not cause the entry of substrates to the binding site to be blocked.

In our initial research on semisynthetic enzymes, we examined briefly the modification of the serine proteinase α-chymotrypsin at a residue on the periphery of the active site Met^{192} (*7*). In most of our work, however, we have modified a residue directly at the active site. The cysteine proteinase papain appeared to be an excellent candidate for this type of modification. Both the x-ray structure of the enzyme (*8–11*) and solution studies with peptide substrates have shown that papain contains an extended groove in the vicinity of the active site residue Cys^{25}. Therefore, it appeared possible that the sulfhydryl group of this residue could be alkylated with a coenzyme analog, while the binding region for potential substrates would remain accessible. Not only would the hydrolytic activity of papain be lost when the cysteine residue would be modified and thus allow the facile monitoring of the modification reaction, but also the introduction of the coenzyme analog might permit a potential substrate to bind.

The choice of a suitable coenzyme analog to act as a modifying agent was based primarily on three criteria.

1) The coenzyme should have the potential to act as a catalyst when bound to an enzyme active site without a requirement for specific functional groups of amino acid residues in the enzyme to participate in the catalytic act.

2) Model building should indicate that the placement of the coenzyme analog is compatible with the spatial requirements of the enzyme template. In other words, there should be a reasonable likelihood that the coenzyme analog, when covalently bound to the active site, would remain in close proximity to the substrate binding site without blocking it.

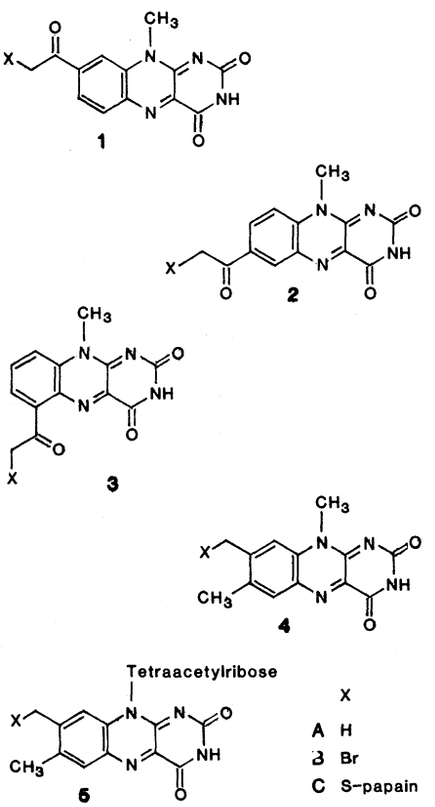
3) Model building should indicate that the covalently bound coenzyme should be capable of interacting with a potential substrate in a productive fashion.

In principle, future research on semisynthetic enzymes may not require adherence to the first criterion mentioned for the choice of the coenzyme analog modifying agent. However, in the early phases of this research, it was questionable whether appropriate modification with coenzyme analogs at enzyme active sites could lead to effective enzymes with new catalytic activities. Therefore, it seemed prudent not to try to build complex cases where the precise geometry of the interaction of functional groups on the enzyme with the coenzyme might be crucial to the development of a successful catalyst.

Flavins were chosen as the modifying agents used in the preparation of the first semisynthetic enzymes because of their known general catalytic versatility. Even model flavins can be quite effective catalysts, and therefore it seemed likely that flavoenzymes could be generated by chemical modification of an enzyme like papain without a requirement for the involvement of specific amino acid functional groups in the enzyme for the catalytic act to occur. Furthermore, flavins are known to catalyze many diverse transformations, suggesting that the preparation of semisynthetic flavoenzymes with different types of catalytic activity would be a distinct possibility.

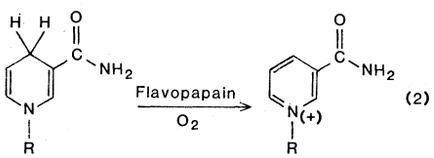
The design of specific flavin analogs for use in the preparation of a semisynthetic enzyme was based on the x-ray diffraction studies of covalent papain-inhibitor complexes by Drenth and colleagues (*12*). These papain derivatives were obtained from the reaction of chloromethyl ketone peptide substrate analogs with the sulfhydryl of Cys^{25}. In each case the carbonyl oxygen of what had originally been the chloromethyl ketone group was positioned near two potential hydrogen-bond donating groups, the backbone NH of Cys^{25} and a side chain NH of Gln^{19}. We thought that building in the possibility of a similar interaction in the case of the flavin modifying agents might serve to constrain the covalently bound flavin moiety to the interior of the enzyme near the substrate binding site. The flavopapains **1C** to **3C**, prepared by alkylation of the sulfhydryl of Cys^{25} and having the potential for this interaction, were constructed according to the second criterion for the choice of the coenzyme modifying agent, namely, that the structure of the coenzyme analog be closely compatible with the geometry of the enzyme template. In contrast, flavopapains **4C** and **5C** do not have a carbonyl group attached to the flavin ring system near the alkylation site and, therefore, could not be held via hydro-

Summary. New active sites can be introduced into naturally occurring enzymes by the chemical modification of specific amino acid residues with the use of appropriately designed coenzyme analogs. The resultant semisynthetic enzymes can have catalytic activities very different from those of the corresponding native enzymes. For example, papain has been converted into a highly effective oxidoreductase by covalent modification of the sulfhydryl group of the active site cysteine residue (Cys^{25}) with flavins such as 8-bromoacetyl-10-methylisoalloxazine. Thus, it is now possible to enhance the catalytic versatility of existing enzymes through the process of "chemical mutation" of the active site.



gen bonding to the active site in the manner proposed for flavopapains **1C** to **3C**. In view of this difference, there may not be a driving force for positioning the flavin moiety in flavopapains **4C** and **5C** near the substrate binding region. On this basis the efficiency of flavopapains **4C** and **5C** as enzymatic catalysts would not be expected to be particularly high. As shall be seen, our experimental work demonstrated the correctness of these predictions.

The oxidation of N^1-alkyl-1,4-dihydronicotinamides by the flavopapains **1C** to **5C** (Eq. 2) is the process that we have examined most carefully. Our model building suggested that various N^1-alkyl-1,4-dihydronicotinamides could be comfortably ensconced within the binding pocket of the semisynthetic enzymes.



Furthermore, in at least some instances, such as flavopapains **1C** and **2C**, the dihydronicotinamide substrates could be placed in close proximity to the flavin group, indicating that hydrogen transfer might be facilitated.

Kinetic and Stereochemical Studies on the Flavopapains

The kinetic data for the oxidation of dihydronicotinamides by flavopapains **1C** to **5C** were measured primarily under aerobic conditions with excess substrate. Under these conditions the accumulation of the dihydroflavin product was not observed for **2C**, **4C**, or **5C**. However, recent findings with the species **1C** indicate that even under aerobic conditions the buildup of the dihydroflavin species produced by reduction of the flavin ring is readily observed (*6*). The general scheme which is postulated to apply to the oxidation of the dihydronicotinamides by flavopapains **1C** to **5C** is illustrated in Eqs. 3A and 3B. For those cases where no buildup of dihydroflavins is seen under aerobic conditions, the rate of formation of nicotinamide is independent of oxygen (that is, $k_o[O_2] >> k_{cat}$). Since dihydroflavin buildup is observed aerobically in the case of flavopapain **1C** under readily accessible conditions, the effect of oxygen on the rate of nicotinamide formation cannot be neglected. If we analyze the kinetics of reaction under aerobic conditions according to the rate expression shown in Eq. 3C, the meaning of the apparent k_{cat} and K_m (Michaelis constant) values will depend on the relative rates of the formation of dihydroflavin in the enzymatic reduction step and of its reaction with oxygen.

Table 1. Kinetic parameters for the oxidation of dihydronicotinamides by 7-acetylflavopapain **2C** and 7-acetylflavin **2A**.

Substrate*	Parameters for reactions			
	Enzymatic†			Model
	K_m (M)	k_{cat} (sec^{-1})	k_{cat}/K_m (M^{-1} sec^{-1})	k_2 (M^{-1} sec^{-1})
NBzNH	1.9×10^{-4}	0.64	3,370	185
NEtNH	1.3×10^{-4}	0.72	5,500	853
NPrNH	1.0×10^{-4}	0.81	8,100	845
NHxNH	0.42×10^{-4}	0.44	10,500	843

*NBzNH, N^1-benzyl-1,4-dihydronicotinamide; NEtNH, N^1-ethyl-1,4-dihydronicotinamide; NPrNH, N^1-propyl-1,4-dihydronicotinamide; NHxNH, N^1-hexyl-1,4-dihydronicotinamide. †Rate constants for the enzymatic reaction were measured at 25°C in 0.1M tris-HCl containing 0.001M EDTA (or in buffer solutions passed through Chelex-100), pH 7.5, 0 to 5 percent ethanol (by volume).

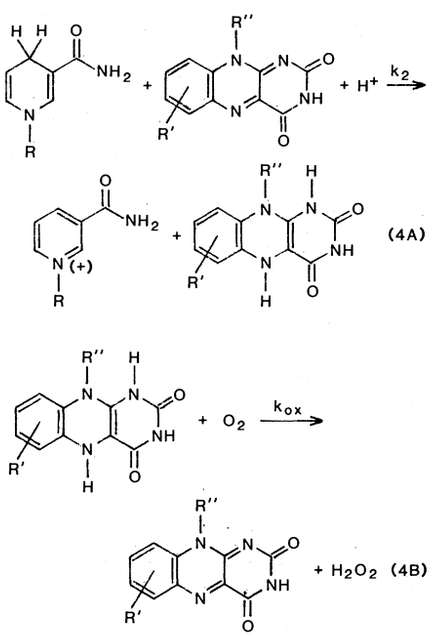
$$E_{ox} + NRNH \underset{K_m}{\rightleftharpoons} ES \xrightarrow{k_{cat}} EH_{2red} + NRN \quad (3A)$$

$$EH_{2red} + O_2 \xrightarrow{k_o} E_{ox} + H_2O_2 \quad (3B)$$

$$v = \frac{k_{cat}\,[E]_0\,[NRNH]}{K_m + [NRNH]} \quad (3C)$$

E_{ox} is the oxidized form of flavopapain, EH_{2red} is the reduced form of flavopapain, ES is the Michaelis complex, NRNH is dihydronicotinamide, NRN is nicotinamide, and k_o and k_{cat} are the rate constants.

In order to assess the effectiveness of the catalytic action of the semisynthetic enzymes produced by covalently attaching flavins **1B** to **5B** to papain's active site, we also studied the kinetics of the model reactions illustrated in Eq 4. Under aerobic conditions and when the N^1-alkyl-1,4-dihydronicotinamide was present in substantial excess (usually, however, at concentrations appreciably less than 0.01M), pseudo first-order kinetics were seen for the model reactions. Under these circumstances, since the pseudo first-order rate constant k_{obs} depends directly on the flavin concentration, the second-order rate constant for the reaction is easily calculated (Tables 1 and 2).



Flavopapains 4C and 5C. In the oxidation of N^1-alkyl-1,4-dihydronicotinamides, flavopapains **4C** and **5C** exhibited a practically insignificant (threefold) rate enhancement over the corresponding reactions of the model compounds **4A** and **5A** and did not display saturation kinetics (*1*). The flavins in the modified enzymes **4C** and **5C** are attached to papain via a thioether bridge. As already mentioned, unlike the species **1C** and **2C**, they do not contain a carbonyl function attached to the flavin at position 8 of the ring system. Since the carbonyl group is predicted to be involved in properly aligning the cofactor relative to the substrate in the enzyme active site, it is not surprising that **4C** and **5C** are poor catalysts.

Flavopapain 2C. Flavopapain **2C** produced from the alkylation of the Cys^{25} residue of papain by the 7-bromoacetyl-substituted flavin **2B** was the first effective semisynthetic enzyme to be prepared (*1, 2*). In the oxidation of N^1-alkyl-1,4-dihydronicotinamides by **2C**, the k_{cat}/K_m values seen are one to two orders of magnitude larger than the second-order rate constants observed for the corresponding model reactions. Furthermore, under conditions of substrate in excess, the semisynthetic enzyme **2C** exhibits saturation kinetics even at a relatively low substrate concentration, a phenomenon not observed in the corresponding model reactions of compounds like **2A**.

Table 1 lists the rate parameters for the oxidation of dihydronicotinamides by flavopapain **2C** and by the model flavin 7-acetyl-10-methylisoalloxazine, **2A**. For comparison, the kinetic parameters in the cases of five naturally occurring flavoenzymes are given in Table 3.

Table 2. Kinetic parameters for the oxidation of dihydronicotinamides by flavopapain **1C** and 8-acetylflavin **1A**. The measurements were made at *p*H 7.5 in 0.1*M* tris-HCl, 0.1 m*M* EDTA containing 0.1 percent ethanol at 25°C.

Substrate	Parameters for reactions			
	Enzymatic			Model
	K_m (M)	k_{cat} (sec^{-1})	k_{cat}/K_m (M^{-1} sec^{-1})	k_2 (M^{-1} sec^{-1})
NBzNH	2.7×10^{-6}	0.093	33,800	170
NPrNH	0.81×10^{-6}	0.048	58,700	878
NHxNH	0.12×10^{-6}	0.067	570,000	917
NADH	340×10^{-6}	0.0073	21	5

It can be seen that the rate constant k_{cat}/K_m for the oxidation of N^1-hexyl-1,4-dihydronicotinamide by flavopapain **2C** is larger than the corresponding rate constant for the oxidation of reduced nicotinamide adenine dinucleotide (NADH) by old yellow enzyme and is comparable to the value of k_{cat}/K_m displayed by glucose oxidase. However, the semisynthetic enzyme's efficiency as a catalyst is somewhat lower than two of the other naturally occurring flavoenzymes shown and much lower than that seen for NADH dehydrogenase. Our results then show that flavopapain **2C**, while not an exceptional catalyst, is a moderately effective flavoenzyme, comparable in activity to a number of the naturally occurring enzymes.

Although an extensive search for the best substrate has not been carried out, some trends in the selectivity toward substrates exhibited by **2C** are nevertheless apparent. Flavopapain **2C** contains an extended hydrophobic binding region. Therefore, it is not surprising that there is an increase in k_{cat}/K_m as the N^1-alkyl group of the dihydronicotinamide increases in chain length. It is probably for the same reason that NADH, having a relatively hydrophilic N^1 substituent, is a very poor substrate for **2C**.

Flavopapain 1C. Flavopapain **1C** is the most efficient semisynthetic enzyme constructed to date (*5*). It can show rate enhancements of nearly three orders of magnitude relative to the corresponding model reactions and displays saturation kinetics in the oxidation of dihydronicotinamides under substrate in excess conditions. The rate parameters for the oxidation of the dihydronicotinamides by flavopapain **1C** are illustrated in Table 2. The k_{cat}/K_m for the oxidation of N^1-hexyl-1,4-dihydronicotinamide by **1C** is either larger than or equal to the corresponding rate parameter for four of the five naturally occurring flavoenzymes listed in Table 3. Indeed, this semisynthetic enzyme approaches the activity displayed by all but the most efficient flavin-containing oxidoreductases known.

Flavopapain 3C. In marked contrast to flavopapains **1C** and **2C**, flavopapain **3C** is an extremely poor catalyst for the oxidation of the N^1-alkyl-1,4-dihydronicotinamides (*6*). The k_{cat}/K_m value for the oxidation of N^1-benzyl-1,4-dihydronicotinamide by **3C**, for example, is 41 M^{-1} sec^{-1} at *p*H 7.5 and 25°C, a value less than the second-order rate constant, 64 M^{-1} sec^{-1}, seen for the corresponding model reaction with flavin **3A**. The enormous difference between the catalytic behavior of flavopapain **1C**, an excellent oxidoreductase, flavopapain **2C**, a moderately effective catalyst, and flavopapain **3C**, a poor catalyst, illustrates the dependence of the enzymatic catalytic efficiency on the proper positioning of the isoalloxazine moiety in the active site. Clearly, the catalytic efficiency of the semisynthetic enzyme produced by chemical modification of the active site of an enzyme is exquisitely sensitive to the proper design of the new catalytic group introduced.

Table 3. Kinetic parameters for several naturally occurring flavoenzymes.

Enzyme	Source	K_m (M)	k_{cat} (sec^{-1})	k_{cat}/K_m (M^{-1} sec^{-1})
NADH-specific FMN oxidoreductase (*13*)	B. Harveyi	47.5×10^{-6}	15.5	3.26×10^5
NADPH-specific FMN oxidoreductase (*13*)	B. Harveyi	40.0×10^{-6}	34.0	8.50×10^5
Old yellow enzyme (*14*)	Yeast	1100×10^{-6}	0.67	6.1×10^2
NADH dehydrogenase (*15*)	Bovine heart			$\sim 10^8$
Glucose oxidase (*16*)				1.05×10^4

Anaerobic Studies and Their Mechanistic Consequences

While we have not, as yet, focused on the mechanisms by which the flavopapains react with their substrates in oxidation-reduction reactions, some information about the pathway followed by dihydronicotinamides has come from studies under anaerobic conditions with flavopapain **2C**. Stopped-flow spectrophotometry was used to study the reaction of flavopapain **2C** with *N*-benzyl-1,4-dihydronicotinamide under anaerobic conditions. When the substrate was present in excess, the reaction displayed biphasic kinetic behavior. We calculated the apparent rate constants for each phase from the experimental data, assuming consecutive first-order kinetics and interpreting the results according to the pathway shown in Eq. 5.

$$E_{ox} + NBzNH \underset{K_s}{\rightleftharpoons} ES \xrightarrow{k_2} ES' \xrightarrow{k_3} EH_{2red} + NBzN \quad (5)$$

where NBzNH is N^1-benzyl-1,4-dihydronicotinamide.

A logical explanation for the biphasic kinetic phenomena is that a labile intermediate is formed during the course of the reaction. This intermediate is shown as ES′ in Eq. 5. The collapse of ES′ to the product corresponds to the slower phase of the reaction, and the apparent first-order rate constant calculated for this phase does not show a dependency on the substrate concentration. The formation of the intermediate corresponds to the initial, faster phase of the reaction.

The flavin moiety in **2C** exhibits an ultraviolet-visible spectrum which deviates from that of the corresponding free flavin. There is a significant long wavelength absorption present in the spectrum of **2C** (with tailing beyond 600 nm) which suggests that a charge-transfer interaction may exist between the flavin and an aromatic amino acid in the active site. This kind of long wavelength tailing is also seen with the reduced flavopapain-*N*-benzylnicotinamide mixture. However, when the ES′ intermediate is observed by stopped-flow spectral measurements in the oxidation of N^1-benzyl-1,4-dihydronicotinamide under anaerobic conditions, the ultraviolet-visible spectrum seen contains far less long wavelength tailing than either the starting flavopapain or the reduced flavopapain-product mixture. This suggests that the charge-transfer complex between the flavin moiety and the aromatic amino acid is disrupted prior to the redox reaction with NBzNH. Although alternative explanations have been examined (*2*),

Table 4. Oxidation of NADH and deuterated NADH derivatives by flavopapain **2C**. Product ratios and rate parameters.

NADH derivative	[4-^{2}H]-NAD$^+$/NAD$^+$	k_{cat}/K_m (M^{-1} sec^{-1})
NADH		68.1
[4A-^{2}H]NADH	0.47	17.3
[4B-^{2}H]NADH	7.33	43.6
[4AB-$^2H_2$]NADH		3.2

these considerations led us to propose the pathway described below for the oxidation of dihydronicotinamides by the related enzyme **1C**.

Figure 1a represents the resting state of the flavoenzyme **1C**. The carbonyl group of the acetyl substituent on the flavin ring system is within hydrogen bonding distance of the backbone NH of Cys^{25} and the side chain NH of Gln^{19}. The flavin entity itself is participating in a charge-transfer complex with the indole side chain of Trp^{26}.

According to our analysis, the initial fast kinetic phase seen in the anaerobic oxidation of N^1-benzyl-1,4-dihydronicotinamide corresponds to a rapid formation of the Michaelis complex illustrated in Fig. 1b; it is followed by generation of the ES′ intermediate illustrated in Fig. 1c. The dihydronicotinamide substrate is bound within the long hydrophobic cavity of the enzyme (Fig. 1b). In the ES′ intermediate (Fig. 1c), the charge-transfer complex existing between the flavin moiety of flavopapain **1C** and an aromatic amino acid in the enzyme's active site (presumably Trp^{26}) is disrupted. The disruption of this complex should lead, as a consequence, to the observed decrease in the long wavelength absorption seen for the oxidized enzyme. In other words, in the reaction of flavopapain **2C** with N^1-benzyl-1,4-dihydronicotinamide the k_2 step seen kinetically corresponds to the formation of the ES′ species illustrated in Fig. 1c in which the flavin moiety has moved to a distinctly different environment from the one it had occupied in the Michaelis complex ES shown in Fig. 1b. In the step leading from the structure of Fig. 1b to that of Fig. 1c, the flavin remains in the oxidized state while the substrate dihydronicotinamide remains reduced. After the realignment of the flavin moiety has taken place giving ES′, the redox reaction in which hydrogen transfer occurs from the dihydronicotinamide to the flavin takes place in the k_3 step, which does not show a rate dependence on the concentration of the substrate.

The postulated formation of the ES′ intermediate (Fig. 1c) also could explain the relative kinetic behavior of flavopapains **1C**, **2C**, and **3C**. Model building indicates that, when the flavin attached to the enzyme via position 8 of the ring system (that is, **1C**) undergoes the rotation step leading to ES′, the N-5 atom of the flavin ring system is in a highly favorable alignment to receive a hydride or hydride equivalent from the substrate. However, when the flavin is attached to the enzyme through position 7 of the ring system (**2C**), this orients the N-5 atom in a manner not quite as advantageous as that in the 8-substituted species **1C**. Finally, the N-5 atom in the 6-acetyl substituted flavin species **3C** is far removed from where we expect the reactive position of the bound substrate to be. For this reason, the low catalytic efficiency of flavoenzyme **3C** is understandable. However, since we have not as yet carried out extensive anaerobic kinetic studies on the reactions of N^1-alkyl-1,4-dihydronicotinamides with the flavopapains **1C** and **3C**, the hypothesis just discussed must be regarded as reasonable but not firmly established. Indeed, our results indicate, as already described, that under aerobic conditions reduction of the flavin moiety of flavopapain **1C** by various N^1-alkyl-1,4-dihydronicotinamides in excess proceeds rapidly relative to the subsequent reaction of the reduced flavin with oxygen to regenerate the oxidized flavin species. This contrasts with the case of flavopapain **2C** and underscores the importance of carrying out detailed anaerobic kinetic comparisons between the behavior of flavopapains **1C** and **2C**.

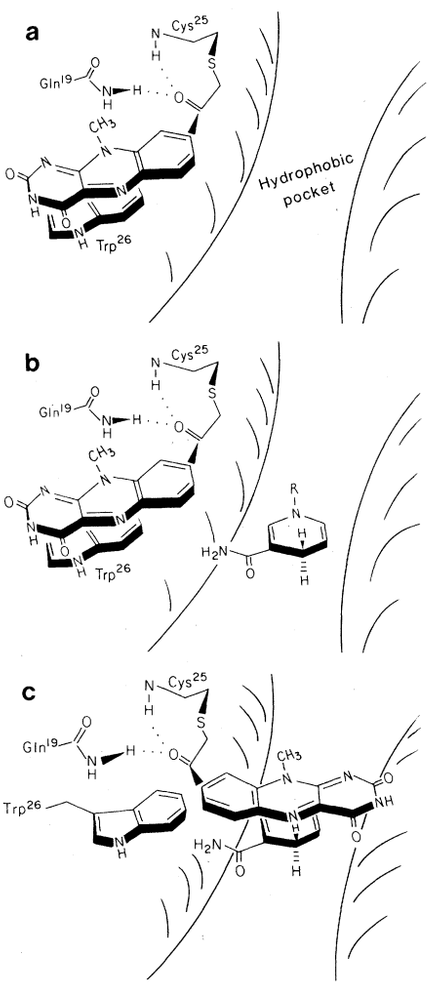
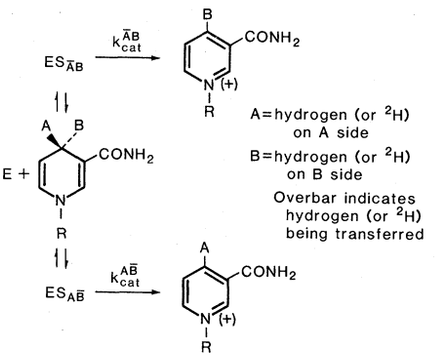


Fig. 1. (a) Active site of the semisynthetic enzyme **1C**. The acetyl side chain of the flavin moiety is hydrogen bonded to the Gln^{19} and Cys^{25} backbone. The flavin is participating in a charge transfer complex with Trp^{26}. (b) Michaelis complex. The dihydronicotinamide is embedded within the hydrophobic groove of the flavoenzyme. (c) ES′ intermediate. The flavin-Trp^{26} charge-transfer complex has been disrupted and the flavin now lies directly over the nicotinamide substrate. The pro-R hydrogen is shown as the species being transferred to the N-5 position of the flavin. This corresponds to the same transfer preference found for the oxidation of labeled NADH by flavopapain **2C** (*3*).



Scheme 1. Kinetic scheme for hydrogen transfer from the A and B faces of the dihydronicotinamide.

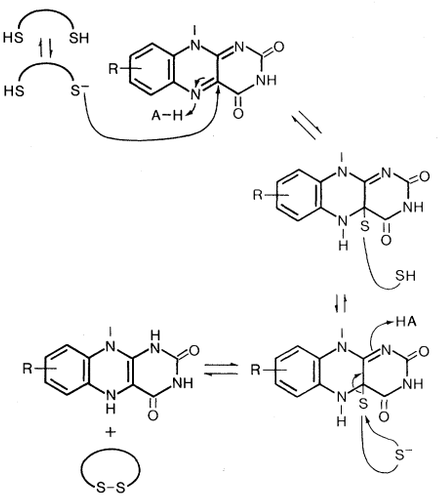
Stereochemical Studies

In view of the asymmetry of the environment of the binding groove of the flavopapains, it is reasonable to expect selectivity in the abstraction of hydrogen from the C-4 prochiral center of the N^1-alkyl-1,4-dihydronicotinamides. Furthermore, it was also anticipated that the semisynthetic enzymes would be able to discriminate between the enantiomers of various dihydronicotinamides and related derivatives containing chiral centers. Considerable experimental support has been obtained for both of these predictions.

The stereochemical consequences of hydrogen transfer from the dihydronicotinamide substrate to the flavin moiety can be elucidated with the use of 1,4-dihydronicotinamides stereospecifically labeled at the C-4 position with deuteri-

um (*17*). However, when our stereochemical work was undertaken, there were no methods available to label stereospecifically model compounds like N^1-alkyl-1,4-dihydronicotinamides in a simple fashion. Consequently, it was necessary to study the reaction of flavopapain with the relatively poor substrate NADH which can be labeled stereospecifically with deuterium at either the 4A or 4B positions (scheme 1). The results of experiments in which flavopapain **2C** was the catalyst for the oxidation of NADH and the corresponding deuterated derivatives are shown in Table 4.

Flavopapain **2C** exhibits a substantial preference for abstraction of the 4A (pro-R) hydrogen of NADH. The stereoselectivity exhibited by flavopapain **2C** is presumed to be the consequence of a difference in the rates of hydrogen transfer from the A and B sides of the 1,4-dihydronicotinamide ring as illustrated in scheme 1. It should be mentioned here that a complicating factor in our study of the oxidation of deuterated NADH derivatives is the possibility that the product NAD^+ and the reactant NADH might undergo nonstereospecific exchange of the C-4 hydrogen (*18*). While we used NADH concentrations less than 0.5 m*M* in order to minimize exchange under our reaction conditions, there is a possibility that such exchange might still complicate the product ratio results shown in Table 4. Ideally, by a calculation combining the results of the rate measurements with the various dihydronicotinamides (undeuterated, monodeuterated, and dideuterated) and the results of product determination on the amount of hydrogen or deuterium transfer, the ratio for the rate of hydrogen transfer from the A side to that from the B side could be calculated. However, a meaningful solution was not obtained in this way, possibly because of complications from the nonstereospecific exchange of the product NAD^+ and the reactant NADH species. As an alternative, the ratio of the rate constants for hydrogen transfer from the A face to the B face was estimated from the relative values of $(k_{cat}/K_m)_{H\bar{D}}$ and $(k_{cat}/K_m)_{D\bar{H}}$. (The hydrogen or deuterium being transferred is denoted by the overbar.) Our results indicate that hydrogen transfer from the A face occurs at a rate approximately sevenfold higher than the corresponding transfer of hydrogen from the B face.



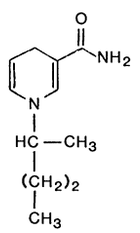
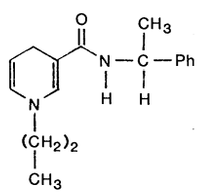
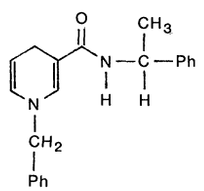
Scheme 2. Postulated mechanism of flavin-catalyzed oxidation of dithiols.

In the case of flavoenzyme **1C** its ability to discriminate between enantiomers of dihydronicotinamide containing chiral centers was also studied briefly. The results of these experiments are provided in Table 5 (*6*).

The enantiomers of the 1,4-dihydronicotinamide **6** which possess a chiral center at the secondary carbon of the alkyl substituent attached to the N^1-position are oxidized at comparable rates. Model building suggests that the substrate can orient its N^1-substituent into the hydrophobic groove of the enzyme and, thus, away from the active site where hydride transfer occurs. If this is, indeed, the case, then it is not surprising that flavopapain **1C** fails to distinguish between enantiomeric dihydronicotinamides which contain the chiral center on the N^1-substituent.

In contrast, when the kinetic behavior for the oxidation of substrates **7** and **8** was determined, appreciable chiral discrimination was observed. In both cases the L isomer reacts faster than its D counterpart (as judged by k_{cat}/K_m) by a factor of approximately 2. Clearly, studies on the chiral selectivity of the flavopapains have not yet been extensive. However, the findings with flavopapain **1C** which shows some selectivity in discriminating between the D and L isomers of compounds **7** and **8**, even though these compounds do not have the chiral center at the reactive function, and the observation with flavopapain **2C** that there is approximately a sevenfold preference for removal of the 4A hydrogen over the 4B hydrogen at the prochiral center are very encouraging.

Table 5. Kinetic parameters for the oxidation by flavopapain **1C** of dihydronicotinamides containing chiral centers. Kinetic measurements were carried out at *p*H 7.5 and 25°C.

	Substrate	Isomer	k_{cat} (sec^{-1})	K_m (*M*)	k_{cat}/K_m (M^{-1} sec^{-1})	Ratio of rate constants L/D
6		D/L	0.052	3.8×10^{-7}	137,000	1
7		D	0.09	19×10^{-6}	5,100	
		L	0.07	6×10^{-6}	12,000	2.4
8		D	0.08	31×10^{-6}	2,200	
		L	0.08	22×10^{-6}	3,800	1.7

Oxidation of Dithiols by Flavopapain 2C

The oxidation of dithiols by flavins has been studied extensively. The generally accepted mechanism (scheme 2) involves a rate determining nucleophilic

attack of a thiolate ion on the C-4A position of the flavin ring (*19, 20*). In our laboratory the oxidation of dithiothreitol (DTT), *dl*-dihydrolipoic acid, and *dl*-dihydrolipoamide by flavopapain **2C** and by the corresponding flavin **2A** has been investigated (*4*), and the kinetic results obtained are shown in Table 6.

In the cases of all three dithiols, the semisynthetic enzyme reacts at a rate faster than that of the corresponding model reaction (comparing k_{cat}/K_m for the enzymatic reaction to the second-order rate constant, k_2, for the model reaction). Furthermore, in spite of the lack of stereoselectivity for the enzymatic reactions, the rate enhancements seen in these cases increases as the hydrophobicity of the substrate increases. This trend, similar to that observed in the oxidation of dihydronicotinamides, is undoubtedly a consequence of the presence of the hydrophobic binding groove in the semisynthetic flavoenzyme which helps bring the reacting dithiols to the proximity of the flavin group.

Table 6. Kinetic parameters for the oxidation of dithiols by flavopapain **2C** and 7-acetylflavin **2A**.

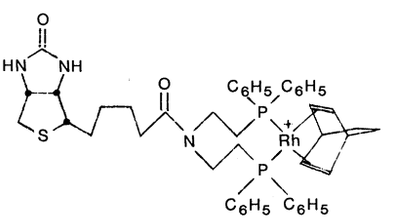
Substrate	Reaction		Rate enhancement
	Enzymatic k_{cat}/K_m (M^{-1} sec^{-1})	Model k_2 (M^{-1} sec^{-1})	
Dithiothreitol*	3.86	0.99	3.9
Dihydrolipoic acid†	6.70	0.84	8.0
Dihydrolipoamide‡	21.0	1.21	17.4

*Rate constants were measured at 25°C at *p*H 7.5. †Same conditions as for * except *p*H 7.3. ‡Same conditions as for * except with 3 to 6 percent dimethyl sulfoxide (by volume).

Other Ways to Construct Semisynthetic Enzymes

In 1966 the conversion by chemical methods of the serine residue in the active site of subtilisin to a cysteine residue was reported by Neet and Koshland (*21, 22*) and by Polgar and Bender (*23, 24*). The resultant "thiolsubtilisin" did not possess proteinase activity although a number of naturally occurring hydrolytic enzymes like papain contain histidine and cysteine as active site residues. Indeed, the semisynthetic species thiolsubtilisin showed significant activity as a hydrolytic catalyst against only the most highly activated substrates, such as nitrophenyl esters and acyl imidazole derivatives. Since the thiolsubtilisin was first reported, "thioltrypsin" (*25*) and "hydroxypapain" (*26*) have been prepared and these semisynthetic enzymes show a similar lack of catalytic activity. The exact reasons for the diminished catalytic activity of these semisynthetic enzymes relative to their natural counterparts are not known. A possible explanation is that in a given enzyme active site environment there is a requirement that the acidic and basic catalytic groups have just the right difference in their ionization constants in order to maximize the rates of proton transfer required for the catalytic act to occur efficiently. In other words, while the ionization constants of the sulfhydryl of cysteine and the imidazole of histidine may have just the right separation in the active site environment of papain, this may not be the case in the environment of the active site of thiolsubtilisin.

In another approach to semisynthetic enzymes, Wilson and Whitesides have reported the use of the biotin binding protein, avidin, as a chiral template (*27*). The complex **9** is a moderately active hydrogenation catalyst that does not exhibit any enantioselectivity. However, in the presence of avidin, **9** catalyzed the hydrogenation of α-acetamidoacrylic acid to (*S*)-*N*-acetylalanine, giving a 34 percent enantiomeric excess of this isomer.



Royer has attempted to generate semisynthetic enzymes from immunoglobulins (*28*). The specificity of an antibody for its antigen or hapten is well known. Royer utilized this specificity to label the binding site of antibodies to a dinitrophenyl hapten with a dinitrophenyl derivative of histidine *p*-nitrophenyl ester. Unfortunately, the modified proteins obtained failed to display any rate enhancement for the catalysis of the hydrolysis of *p*-nitrophenyl acetate. This lack of catalytic activity was ascribed to a misorientation of the bound substrate.

Finally, recombinant DNA technology has provided a means to obtain site-specific mutations at the active site of an enzyme. Restriction enzymes are used to excise specific gene segments, and then chemically synthesized oligonucleotides containing the information for the desired amino acid substitution are introduced. This methodology has resulted in the expression of a β-lactamase in which the active site serine has been replaced by a cysteine. The resultant "thiol-β-lactamase" exhibits reduced but detectable activity (*29*). The use of site-specific mutagenesis in combination with the "chemical mutation" methodology described in this article should increase enormously the range of possibilities in the design of semisynthetic enzymes.

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