

# Semisynthetic Enzymes: Design of Flavin-Dependent Oxidoreductases

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## Introduction

How do enzymes work? What are the general rules that relate protein structure to catalytic activity? Is it possible to design proteins rationally that mimic the details of enzyme action? The answers to these questions are of great interest to the scientific community. This is easy to understand since few chemical catalysts can achieve the rates and specificities of nature's own catalysts. Our ability to treat enzyme-related diseases, to regulate enzyme-mediated processes with drugs, or to use enzymes themselves as therapeutic agents or industrial catalysts, depends on a fundamental understanding of enzyme action. Furthermore, because many important chemical reactions lack physiological catalysts, the ability to prepare tailored enzymes would extend enormously the range of possible applications for these materials.

As it is not yet possible to design polypeptides with predictable, stable tertiary structures, we have attempted to build new activities into existing enzymes by site-directed mutagenesis. Naturally occurring proteins bind a wide range of ligands in well-defined complexes under mild aqueous conditions and are therefore excellent potential templates for the construction of artificial enzymes. We have utilized both recombinant DNA techniques and chemical methodologies to alter the binding sites of several carefully chosen enzymes. The manipulation of protein structure at the gene level is currently a very exciting and active field of research (Ulmer, 1983). Neverthe-

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Abbreviations: BNAH, *N*-benzyl-1,4-dihydronicotinamide; DCIP, dichloroindophenol; EPPS, 4-(2-hydroxyethyl)-1-piperazinepropane sulphonic acid; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; HNAH, *N*-hexyl-1,4-dihydronicotinamide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,4-diphenyltetrazolium bromide; NAD(H), nicotinamide adenine dinucleotide (reduced form); PNAH, *N*-propyl-1,4-dihydronicotinamide.

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less, in such studies one is limited to changes (substitutions, additions or deletions) that involve the 20-odd natural amino acids. The chemical mutation of enzyme active sites is a complementary strategy that extends the range of possible changes that can be made (Kaiser and Lawrence, 1984). For example, active site residues can be converted by specific chemical reactions into unnatural amino acids. Alternately, a reactive cofactor molecule can be covalently attached to the active site of an enzyme to produce a novel hybrid species that possesses the binding properties of the template and the intrinsic catalytic activity of the prosthetic group. These hybrids, 'semisynthetic enzymes', are the subject of this article.

If the chemical mutation of a given enzyme is to yield a successful catalyst, a number of considerations must be weighed. A suitable reactive derivative of the desired prosthetic group must be synthetically accessible, and the cofactor must fit into the active site of the target enzyme without blocking the binding of potential substrates. The choice of template is therefore critical. Structural information relating to the protein is, of course, invaluable for the design of an efficient semisynthetic enzyme. Knowledge of the location and reactivity of specific amino acid residues in the active site permits the formulation of the best strategies for introducing the prosthetic group in such a way that it will be able to interact productively with bound substrate. Other factors, such as the availability and stability of the template molecule, are also relevant.

In our preliminary evaluation of the chemical mutation strategy we have avoided systems that require rapid proton transfer steps for catalysis. Flavin cofactors have proved to be particularly good prosthetic groups in our work (Kaiser and Lawrence, 1984) although many other cofactors are likely to be successful. Isoalloxazine derivatives are readily synthesized and easily modified. Importantly, they are also good model compounds for the biological cofactors FAD and FMN. In free solution, they catalyse many of the same reactions promoted by naturally occurring flavoenzymes, including oxidation of dihydronicotinamides and thiols and activation of molecular oxygen, albeit with lower rates and selectivities. To date, we have successfully used a series of isoalloxazine derivatives with three templates, papain (EC 3.4.4.10), glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) and haemoglobin, to create efficient flavin-dependent oxidoreductases. These systems are discussed in turn.

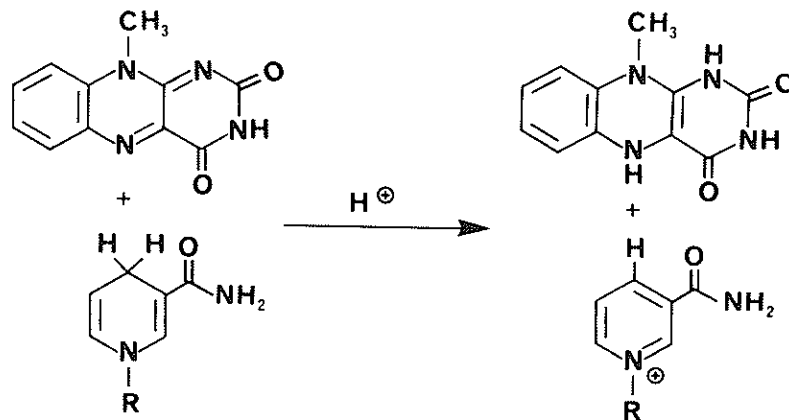
### **Flavopapain**

Early in our research on semisynthetic enzymes we decided to test the consequences of modifying the active site of a proteolytic enzyme with a suitable flavin coenzyme analogue (Levine, Nakagawa and Kaiser, 1977; Levine and Kaiser, 1978). The proteolytic enzyme on which we have done most of our work is papain. We chose this enzyme as a template because its X-ray structure and those of several derivatives are known (Drenth *et al.*, 1968, 1977; Drenth, Kalk and Swen, 1976) and also because the reaction kinetics of the enzyme are quite well understood (Glazer and Smith, 1971).

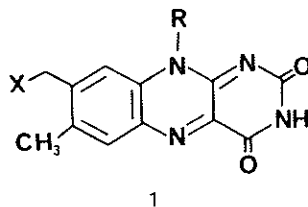
In addition, this enzyme has a uniquely reactive sulphhydryl group (Cys-25) in its active site which was known to be selectively alkylated by a variety of reagents. This cysteine is located in a groove that is about 25 Å (2.5 nm) long, and on the basis of model building it appeared likely that suitable substrate molecules would be able to bind close to a flavin linked to it. We felt that rapid catalysis of a reaction such as the oxidation of a 1,4-dihydronicotinamide might result from such proximity (*Figure 1*). The results that we have obtained to date on several flavopapains provide strong evidence that this hypothesis is correct.

#### SUBSTITUENTS

Because the active site of papain is an open cleft, the manner of attachment of the flavin cofactor to Cys-25 is particularly crucial. In order to elucidate the optimal geometry for linking a flavin to the active site of papain we have prepared a series of isoalloxazine derivatives containing reactive groups capable of alkylating the targeted thiol residue. These compounds have varied both in the nature and in the position of their alkylating functionality which affects their position within the groove. It is essential, for instance, that the covalently bound prosthetic group should remain in the binding site rather than it should flip out on to the protein surface. Obviously, if the flavin were to adopt a conformation which placed it outside the binding pocket, it would be unable to interact with bound substrates in a productive fashion, making effective catalysis impossible. When we examined the active site region of papain we could see that a flavin molecule attached to the Cys-25 residue through a benzyl position, as in the 8 $\alpha$ -substituted structure 1 (*Figure 2*), might not remain in the binding site and might instead move



**Figure 1.** Reduction of flavin by *N*-alkyl-1,4-dihydronicotinamide. In the presence of molecular oxygen the dihydroflavin formed is reoxidized, making the overall reaction catalytic in flavin.



**Figure 2.** 8 $\alpha$ -Methyl-substituted flavin. 1a: X=Br, R=tetraacetylribose; 1b: X=Br, R=CH<sub>3</sub>.

out on to the surface of the protein. Indeed, experiments with flavins related to structure 1 suggested strongly that these compounds did not remain in the binding site. In particular, we found that when we modified papain with 8 $\alpha$ -bromotetraacetylriboflavin (1a) or with 8 $\alpha$ -bromolumiflavin (1b) (*Figure 2*), the flavopapains we obtained did not exhibit saturation kinetics in the oxidation of several dihydronicotinamides such as *N*-benzyl-1,4-dihydronicotinamide and *N*-propyl-1,4-dihydronicotinamide at substrate concentrations of about  $10^{-3}$  M (Levine, Nakagawa and Kaiser, 1977). Also, most importantly, the second-order rate constants which we calculated for the oxidation of the 1,4-dihydronicotinamides by these flavopapains showed no appreciable rate accelerations relative to related reactions catalysed by the structurally analogous free flavins.

On the other hand, flavopapains 2a and 3a (*Figure 3*), which were obtained by alkylation of the active site sulphhydryl by 7 $\alpha$ -bromoacetyl-10-methylisoalloxazine (2b) and by 8 $\alpha$ -bromoacetyl-10-methylisoalloxazine (3b), exhibited saturation kinetics at relatively low dihydronicotinamide concentrations (Levine and Kaiser, 1978; Slama, Oruganti and Kaiser, 1981). Our observations that the flavopapains derived from these  $\alpha$ -halo carbonyl systems were effective oxidants were not surprising in view of the known X-ray diffraction studies of covalent papain-inhibitor complexes resulting from the reaction of chloromethylketone peptide substrate analogues with the sulphhydryl of Cys-25. For the various structures examined, these studies show that the carbonyl group of what had previously been the chloromethylketone group was in close proximity to two potential hydrogen-bond donating groups, the backbone NH of Cys-25 and a side chain NH of Gln-19 (Drenth, Kalk and Swen, 1976). The design of the flavin moieties of flavopapains 2a and 3a was based on the premise that a similar interaction might occur through the presence of the carbonyl moiety of the acetyl groups attached to the flavin ring system. Such interactions might aid in constraining the covalently bound flavin to the interior of the enzyme close to the vicinity of the substrate binding site. Such interactions cannot, however, be the sole factor responsible for efficient catalysis. 6-Acetylflavopapain (4a) (*Figure 3*), despite its carbonyl group, is an ineffective catalyst for the oxidation of any of the substrates examined (Slama *et al.*, 1984).

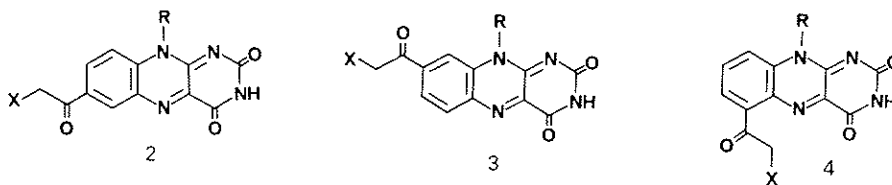


Figure 3. Three flavin-alkylating agents. 2a, 3a, 4a: X=S-papain; 2b, 3b, 4b: X=Br; 2c, 3c, 4c: X=H; 2d, 3d, 4d: X=S-GAPDH.

## KINETICS

We found that the  $k_{cat}/K_m$  value measured in the oxidation of *N*-benzyl-1,4-dihydronicotinamide by 7-acetylflavopapain (2a) under aerobic conditions was approximately 20 times greater than the second-order rate constant for the corresponding oxidation reaction catalysed by model flavin 2c (Kaiser *et al.*, 1980). A survey of the kinetics of the oxidation of various *N*-alkyl-1,4-dihydronicotinamides by 2a showed that the apparent  $K_m$  values for these reactions typically ranged in the region around  $10^{-4}$  M. The compound most rapidly oxidized by 7-acetylflavopapain (2a) in our studies was the *N*-hexyl species that underwent reaction with a  $k_{cat}/K_m$  value of  $10\,500\text{ M}^{-1}\text{ s}^{-1}$  at pH 7.5 and 25°C. On the other hand, bulky polar compounds like NADH are relatively poor substrates for the semisynthetic enzyme. In the case of NADH itself, the  $k_{cat}/K_m$  value is only about  $70\text{ M}^{-1}\text{ s}^{-1}$ . The observed substrate specificity was anticipated, given the hydrophobic nature of the active site cleft of papain. Furthermore, as the binding pocket of papain is chiral, some stereoselectivity was also expected. In fact, we observed a modest preference for transfer of the pro-R hydrogen of NADH to the enzyme-bound flavin (Levine and Kaiser, 1980).

The most effective flavopapain that we have described is species 3a which reacts rapidly with a variety of hydrophobic *N*-alkyl-1,4-dihydronicotinamides. The  $k_{cat}/K_m$  value measured for the oxidation of the best of the substrates examined, *N*-hexyl 1,4-dihydronicotinamide, is in the vicinity of  $10^6\text{ M}^{-1}\text{ s}^{-1}$  in air-saturated buffer at pH 7.5 and 25°C (Slama, Oruganti and Kaiser, 1981; Slama *et al.*, 1984). Comparing this rate parameter with the second-order rate constant for the corresponding oxidation reaction catalysed by the model compound 3c shows that there is at least a  $10^3$ -fold rate acceleration for the enzymatic system. For the oxidation of *N*-benzyl-1,4-dihydronicotinamide by 8-acetylflavopapain (3a) the  $k_{cat}/K_m$  value measured was  $67\,000\text{ M}^{-1}\text{ s}^{-1}$  and for *N*-propyl-1,4-dihydronicotinamide it was  $86\,000\text{ M}^{-1}\text{ s}^{-1}$  under the same conditions. For the *N*-benzyl compound this represented a rate acceleration of nearly 300-fold compared with the oxidation by the model flavin 3c, and for the oxidation of the *N*-propyl compound the rate acceleration was approximately 100-fold.

Using anaerobic conditions and the stopped-flow technique, we have investigated the individual steps in the oxidation of *N*-alkyl-1,4-dihydronicotinamides catalysed by 8-acetylflavopapain (3a) (Radziejewski, Ballou and Kaiser, 1985). Under conditions of substrate in excess at 25°C the reduction

**Table 1.** Rate parameters for oxidation reactions catalysed by 8-acetylflavopapain (3a, *Figure 3*) and 8-acetyl-10-methylisoalloxazine (3c) (Radziejewski, Ballou and Kaiser, 1985)

Substrate	Electron acceptor	3a			3c
		$k_{cat}$ (s <sup>-1</sup> )	$K_m$ ( $\mu$ M)	$k_{cat}/K_m$ (M <sup>-1</sup> s <sup>-1</sup> )	$k_2$ (M <sup>-1</sup> s <sup>-1</sup> )
HNAH*	none†	370	230	$1.64 \times 10^6$	—
	O <sub>2</sub> ‡	0.067	0.12	$5.70 \times 10^6$	917
	MTT	—	—	—	1345
BNAH*	none†	29	471	$61.8 \times 10^3$	—
	O <sub>2</sub> §	0.13	2	$67.4 \times 10^3$	170
	MTT	31.5	540	$58.8 \times 10^3$	210
PNAH*	none†	31	210	$1.79 \times 10^5$	—
	O <sub>2</sub> ‡	0.056	0.65	$86 \times 10^3$	878
	MTT	62.5	1000	$60.9 \times 10^3$	1150

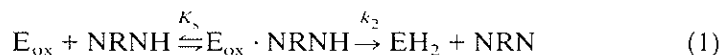
\*HNAH, BNAH and PNAH refer to *N*-hexyl-, *N*-benzyl- and *N*-propyl-1,4-dihydronicotinamide, respectively.

†Anaerobic conditions. Measured at 24.5°C in 67 mM HEPES pH 7.6 with stopped flow spectrophotometer.

‡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.

§Enzymatic rates were measured in oxygen-saturated Tris-HCl, 0.1 mM EDTA pH 7.5 buffer containing the above-mentioned scavenging enzymes.

of the semisynthetic enzyme by *N*-alkyl-1,4-dihydronicotinamides showed pseudo first-order kinetics at two wavelengths, 450 and 530 nm. From plots of the pseudo first-order rate constants which were measured versus the substrate concentration we were able to conclude that saturation conditions could be approached for the oxidation of three dihydronicotinamides, *N*-hexyl-, *N*-benzyl- and *N*-propyl-1,4-dihydronicotinamide. The data we obtained under anaerobic conditions for the reactions of the dihydronicotinamides with flavopapain 3a are summarized in *Table 1* together with some results for the same oxidation reactions under aerobic conditions. The anaerobic results obtained are consistent with the scheme of equation 1. Inspection of *Table 1* shows that the rapidity of the enzymatic reactions reflects to a considerable extent the high values of  $k_2$ , the rate constant for the conversion of the Michaelis complex to the reduced dihydroflavopapain species. In fact, measurements on the *N*-hexyl compound showed that, even with a very efficient stopped-flow instrument, the  $k_2$  value was so large that only an estimate of this rate constant could be calculated.



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

## ACCEPTORS

We also studied the reaction of reduced 8-acetylflavopapain (3a) with oxygen under steady state conditions (Radziejewski, Ballou and Kaiser, 1985). As observed with model dihydroflavins, the reaction of dihydroflavopapain with oxygen shows autocatalytic behaviour and produces superoxide which acts itself in causing further oxidation. Some reduction of the rate of oxidation was consequently observed when superoxide dismutase was added. Nevertheless, oxygen is a relatively poor oxidant for flavopapain. Although hydride transfer from the *N*-alkyl-1,4-dihydronicotinamides to the flavopapain is very rapid, reoxidation of the dihydro form of the enzyme turns out to be about 7–8 times slower than the similar reaction seen with the dihydro form of 8-acetyl-10-methylisoalloxazine (3c). From the data in *Table 1* it can also be seen that, under the turnover conditions used for the aerobic reactions, the reaction of oxygen with the dihydroflavin must be largely rate limiting over the substrate concentration range that we employed. To achieve turnover conditions where the reduction step rather than the oxidation of the dihydroflavin is rate limiting, it is necessary to employ an acceptor that reacts more rapidly than oxygen.

We have therefore explored the ability of various electron acceptors other than molecular oxygen to reoxidize the dihydro form of 8-acetylflavopapain (3a) and the corresponding model system 3c (Radziejewski, Ballou and Kaiser, 1985). Dichloroindophenol (DCIP), coenzyme  $Q_{10}$ , 3-(4,5-dimethylthiazol-2-yl)-2,4-diphenyltetrazolium bromide (MTT) and cytochrome *c* are all excellent electron acceptors in this reaction. The second-order rate constants for oxidation of dihydroflavopapain by dichloroindophenol, coenzyme  $Q_{10}$  and cytochrome *c*, for example, were determined by stopped-flow and found to be in the range  $10^6$ – $10^7$   $\text{M}^{-1}\text{s}^{-1}$ . These values are approximately tenfold slower than those for oxidation of the corresponding non-enzymatic model flavin. In addition, while cytochrome *c* is probably reduced by flavopapain in one-electron steps, we were unable to obtain any evidence for the semiquinone form of the flavoenzyme in the electron transfer step. In any case, all of these compounds are better electron acceptors for the reduced enzyme than molecular oxygen. We found that MTT, in particular, is an excellent acceptor for achieving high turnover rates in practice. The results summarized in *Table 1* show that it is feasible to obtain  $k_{\text{cat}}$  values for the flavopapain-catalysed oxidation of *N*-benzyl- and *N*-propyl-1,4-dihydronicotinamides in the presence of MTT which are similar to the numbers obtained under anaerobic conditions for the reduction of the enzyme by these substrates. Thus, it has been demonstrated that an artificial redox enzyme exhibiting high catalytic efficiency *and* a high turnover number can be constructed from the binding site of a well-characterized hydrolytic enzyme.

## OTHER REACTIONS CATALYSED

We have also shown that flavopapains can mediate the oxidation of thiols to disulphides. 7-Acetylflavopapain (2a) catalysed the oxidation of dithiols, but the rate accelerations seen relative to oxidation by the model flavin

2c were quite modest (Fried and Kaiser, 1981). On the other hand, 8-acetylflavopapain (3a) was found to be an effective catalyst for such reactions. Under anaerobic conditions the  $k_2/K_s$  values for the reduction of the flavopapain by D,L-dihydrolipoamide and D,L-dihydrolipoic acid were 4400 and 3400  $\text{M}^{-1}\text{s}^{-1}$ , respectively (Stewart, Radziejewski and Kaiser, 1986). These rate parameters were 126 and 200 times larger than the second-order rate constants for the corresponding reactions of D,L-dihydrolipoamide and D,L-dihydrolipoic acid with the model compound 8-acetyl-10-methylisoalloxazine (3c). When we used the dye MTT as an electron acceptor, we obtained  $k_{\text{cat}}$  and  $K_m$  values for the oxidation of dihydrolipoamide by flavopapain (3a) which were in approximate agreement with the  $k_2$  and  $K_s$  values, demonstrating that the rate-limiting step of the catalytic cycle is substrate oxidation rather than reoxidation of dihydroflavin.

### Flavo-GAPDH

Many proteins in addition to the proteolytic enzymes are potentially suited for use in constructing semisynthetic enzymes. The enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH), for example, has been extensively studied, and both its primary and tertiary structures are available (Harris and Waters, 1976). In contrast to papain, active GAPDH is tetrameric ( $M_r$  145 000). Each subunit is chemically identical and contains a binding site for both  $\text{NAD}^+/\text{NADH}$  and glyceraldehyde-3-phosphate. The nicotinamide cofactor binds in an open extended conformation similar to that found in liver alcohol dehydrogenase (EC 1.1.1.1). Like papain, GAPDH also contains an essential thiol functionality (Cys-149) in the active site. This group is located near the binding site for the pyridine portion of  $\text{NAD}^+/\text{NADH}$ , and is the putative binding site for glyceraldehyde-3-phosphate. Cys-149 is readily alkylated by a wide range of reagents. Moreover, alkylation does not prevent nicotinamide binding: Rafter and Colowick (1957) showed, for example, that treatment of GAPDH with dichloroindophenol (DCIP) serendipitously converts the enzyme into a diaphorase. Apparently, Cys-149 reacts with DCIP to give a covalent adduct that can be isolated by gel chromatography and subsequently oxidized to a blue quinone with excess DCIP or oxygen (Benitez and Allison, 1973). This enzyme-bound quinone dye is reduced in the presence of NADH in a reaction that occurs at the active site (Benitez and Allison, 1973).

The work of Rafter and Colowick (1957) suggested to us that GAPDH might be a good template for the rational design of flavin-dependent oxidoreductases *selective for NADH* rather than for hydrophobic *N*-alkyl-1,4-dihydronicotinamides. This conclusion was strengthened by model-building studies using the X-ray coordinates of the enzyme (Moras *et al.*, 1975; Biesecker *et al.*, 1977). The active site of the enzyme is large and can sterically accommodate a flavin moiety, even in the presence of bound nicotinamide. Indeed, it seemed likely that bound NADH would be able to transfer a hydride efficiently to a flavin moiety attached to Cys-149, with the *si*-face stereoselectivity of the native enzyme. Unmodified GAPDH binds  $\text{NAD}^+$  in a negatively co-operative fashion, and the possibility of investigat-



ing co-operative interactions in a model system was an additional attractive feature of this system. In contrast to NADH, NADPH is not bound by native GAPDH, so it was not expected to be a good substrate for the semisynthetic enzyme. Similarly, hydrophobic *N*-alkyl-1,4-dihydronicotinamides that had been the best substrates for flavopapain were expected to be poorly oxidized at the polar active site of the flavo-GAPDH. In short, GAPDH seemed ideal for developing semisynthetic flavoenzymes with substrate specificity and stereoselectivity opposite that of flavopapain and therefore an excellent system for evaluating the generality of the chemical mutation strategy for catalyst design.

#### TYPE OF GAPDH

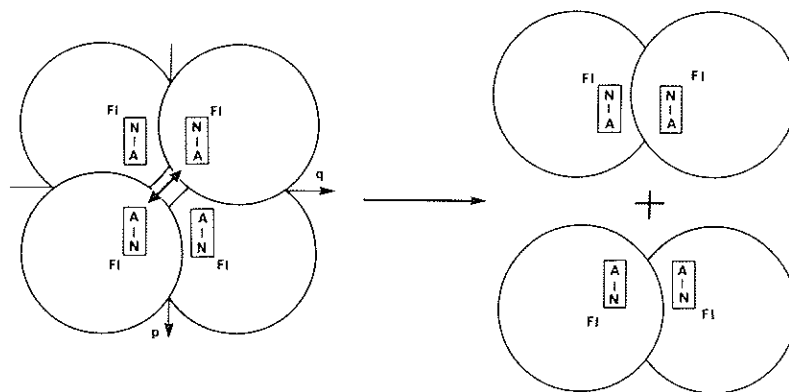
In the cell, GAPDH has a key role in carbohydrate metabolism catalysing the reversible oxidative phosphorylation of glyceraldehyde-3-phosphate with concomitant reduction of  $\text{NAD}^+$  (Harris and Waters, 1976); it is consequently available from a wide range of sources. The existence of natural variants is advantageous, as it makes possible the examination of the relationship between protein structure and the chemical and physical properties of the catalysts we prepare. In our studies to date, we have employed GAPDH from rabbit muscle tissue and from the thermophilic bacterium *Bacillus stearothermophilus*. Available structure data indicate a high degree of homology between the mesophilic and thermophilic enzymes (Biesecker *et al.*, 1977). Nevertheless, the bacterial enzyme is significantly more stable than the muscle protein. It regains full activity following exposure to 1 M urea for up to one hour at 20°C and can be studied at elevated temperatures without loss of activity (Amelunxen, 1967). The rabbit muscle enzyme, on the other hand, rapidly loses activity under such conditions, especially in the absence of  $\text{NAD}^+$  (Harris and Waters, 1976). The thermostability of *B. stearothermophilus* GAPDH is due, in part, to several extra buried ionic bonds (Walker, Wonacott and Harris, 1980). Additional hydrophobic interactions at the subunit interfaces are also important, especially between the so-called S-loops that form the core of the tetramer (Walker, Wonacott and Harris, 1980).

#### STRUCTURE AND PROPERTIES OF 7-ACETYLFLAVO-GAPDH

Normally, GAPDH is isolated with several molecules (2–4) of  $\text{NAD}^+$  bound at the active site (Harris and Waters, 1976). As the presence of this substrate protects the thiol group of Cys-149 from alkylation (Trentham, 1968), it must be removed prior to treatment of the enzyme with the isoalloxazine alkylating agents. This is readily accomplished by chromatographing the holo-enzyme on a column of Cibacron Blue or acid-washed Norit. Apo-GAPDH from rabbit muscle (Hilvert and Kaiser, 1985) and *B. stearothermophilus* (Hilvert, Hatanaka and Kaiser, 1987) reacts readily with 7 $\alpha$ -bromoacetyl-10-methyl-isoalloxazine (2b) at room temperature in aqueous buffer. The resulting semisynthetic flavoenzymes are separated from unreacted isoalloxazine by

gel filtration chromatography on Sephadex G-25, and the modified proteins characterized using standard techniques. Although the rabbit muscle enzyme contains four cysteine groups and the bacterial enzyme two per polypeptide chain, only one flavin molecule is incorporated per subunit. The titratable active-site thiol of the native enzyme is lost after the modification procedure, indicating that alkylation is specific for Cys-149. The substrate specificity and stereoselectivity of these semisynthetic enzymes provides additional, circumstantial evidence for the selectivity of the alkylation protocol, as discussed below.

On the basis of HPLC and gel filtration evidence, initially formed 7-acetylflavo-GAPDH (2d) is tetrameric. The bacterial semisynthetic enzyme, however, subsequently undergoes slow dissociation into dimers in an apparently irreversible process (Hilvert, Hatanaka and Kaiser, 1987). After 20 hours at 4°C, the dimer accounts for more than 80% of the total protein in the sample. The active site of GAPDH is located between two large protein domains, and incorporation of the isoalloxazine moiety presumably acts like a wedge, prizing open the binding pocket and shifting the tetramer-dimer equilibrium towards dissociation. There are relatively few intersubunit contacts across the crystallographic Q-axis, and these are the interactions that are probably lost when the tetramer dissociates (*Figure 4*). The resulting dimeric bacterial flavoprotein would still be held together by extensive  $\beta$ -sheet contacts across the P-axis. In fact, the flavin-containing dimer is stable over a period of many months and can be purified by gel filtration. In contrast, the rabbit muscle flavoprotein is far less robust, losing 40–60% of its activity overnight. Because it must be assayed immediately following



**Figure 4** Proposed model of tetrameric and dimeric 7-acetylflavo-GAPDH. The four subunits are related by the dyad axes shown (the R-axis is perpendicular to the picture plane). The flavin is symbolized by F; NADH is represented by N-A where N corresponds to the nicotinamide and A to the adenosyl portions of the substrate, respectively. The heavy arrow indicates *intersubunit* interactions in the adenosyl binding pocket which ordinarily prevent the binding of NADPH to the enzyme.

preparation, only the tetramer could be studied. It seems likely that the rabbit muscle flavoenzyme also dissociates into dimers. In this case, however, the dimer may be unstable and may undergo further irreversible denaturation, accounting for the observed loss of activity. In addition to different stabilities, 7-acetylflavo-GAPDH (2d) from rabbit muscle and *B. stearothermophilus* show interesting differences in their kinetic profiles, stereoselectivity and substrate specificity. As will be discussed below, many of these individual characteristics can be explained in terms of the quaternary structures of the respective enzymes.

## KINETICS

The oxidation of dihydronicotinamides was used as a model system for evaluation of the kinetic efficiency of these multimeric semisynthetic flavoenzymes. The steady-state kinetic parameters for a number of substrates is given in *Table 2*. Several points deserve comment. First, as expected, NADH is the optimal substrate for both proteins. The data obtained with the rabbit muscle enzyme are readily fit by standard Michaelis–Menten kinetics ( $k_p = k_{cat}$  and  $K_s = K_m$ ) (Hilvert and Kaiser, 1985), but pronounced negative cooperativity is observed in the bacterial case (Hilvert, Hatanaka and Kaiser, 1987). The latter data are fitted very well by equation 2 for a simple sequential interaction model (Segel, 1975). The parameters  $k_p$  and  $K_s$  in this equation are the turnover number and intrinsic dissociation constant, respectively. The interaction factor  $a$  reflects the degree to which the intrinsic dissociation constant changes upon binding the first molecule of substrate. For negatively co-operative systems  $a > 1$ ; for non-cooperative systems the value of  $a$  is 1, and equation 2 reduces to the Michaelis–Menten equation. Although trivial explanations for the apparent co-operativity (a population of heterogeneous active sites) cannot be conclusively ruled out at this time, the fact that reactivity of half of the sites is well documented for GAPDH itself (Levitzky, 1974) suggests that co-operative catalysis by bacterial 7-acetylflavo-GAPDH (2d) is not an unreasonable possibility. Co-operative interactions between the subunits could be transmitted, for example, by conformational changes in the S-loop upon substrate binding. Significantly, non-specific substrates like *N*-propyl-1,4-dihydronicotinamide do not elicit negatively co-operative kinetic profiles with either enzyme.

$$v = \frac{2k_p \left[ \frac{S}{K_s} + \frac{S^2}{aK_s^2} \right]}{[E]_T \left[ 1 + \frac{2S}{K_s} + \frac{S^2}{aK_s^2} \right]} \quad (2)$$

Comparison of the apparent bimolecular rate constant  $k_p/K_s$  with the second-order rate constant  $k_2$  for an appropriate non-enzymatic model system gives the maximum rate acceleration achieved by the enzyme. By this criterion the oxidation of NADH by 7-acetylflavo-GAPDH is highly efficient:

**Table 2.** Steady state parameters\* for the oxidation of 1,4-dihyronicotinamides by 7-acetylflavo-GAPDH from rabbit muscle and *B. stearothermophilus* (Hilvert and Kaiser, 1985; Hilvert, Hatanaka and Kaiser, 1987)

Enzyme	Substrate	$k_p$ ( $s^{-1}$ )	$K_s$ ( $\mu M$ )	$a$	$k_p/K_s$ ( $M^{-1}s^{-1}$ )
Rabbit	NADH	0.0273	25.4	1	1067
	NADPH	0.0061	104.0	1	59
	PNAH	0.409	525.0	1	779
<i>B. stearothermophilus</i>	NADH	0.0647	0.853	52	75 900
	NADPH	0.0391	2.23	4	17 600
	PNAH	0.0457	23.2	1	1970

\* Determined in air-saturated aqueous buffer (50 mM EPBS or 50 mM Tris-HCl, pH 7.5) containing the scavenging enzymes described in the legend for *Table 1*.

at 25°C in air-saturated buffer,  $k_2$  for the oxidation of NADH by 7-acetyl-10-methylisalloxazine (2c) is only  $12.9 M^{-1}s^{-1}$ . The rate enhancement observed in the case of the rabbit muscle enzyme under these conditions is therefore 83-fold, and for the bacterial protein it is nearly 6000-fold (*see Table 2*). The latter value is 10 times larger than that obtained in the oxidation of *N*-hexyl-1,4-dihyronicotinamide by flavopapain (Slama *et al.*, 1984). Thus, with careful choice of protein template the chemical mutation strategy for catalyst design is generalizable. In addition, the dimeric semisynthetic flavoenzyme derived from *B. stearothermophilus* is remarkably thermostable. Unlike rabbit muscle 2d which rapidly denatures at high temperatures, the bacterial dimer is stable enough at 55°C to be assayed. The kinetic parameters for NADH oxidation obtained at this temperature are:  $k_p = 0.262 s^{-1}$ ,  $K_s = 4.66 \mu M$ ,  $k_p/K_s = 56 200 M^{-1}s^{-1}$  and  $a = 27$ . The second-order rate constant for the non-enzymatic model reaction increases to  $23.1 M^{-1}s^{-1}$  at 55°C, so the net enzymatic rate acceleration at low substrate concentrations is still greater than three orders of magnitude. Significantly, the magnitude of  $k_p/K_s$  for bacterial 7-acetylflavo-GAPDH is in the range of  $k_{cat}/K_m$  values observed for naturally occurring flavoenzymes that oxidize NADH (Kaiser and Lawrence, 1984).

As in the case of flavopapain, turnover of 7-acetylflavo-GAPDH (2d) under aerobic conditions is limited by the rate of reoxidation of the dihydroflavin generated during each catalytic cycle. Consequently, the value of  $k_p$  measured depends explicitly on oxygen concentration and does not reflect the true efficiency of the hydride transfer step. To determine the rates of reduction of the flavin in the enzyme-substrate complex, rapid mixing techniques under anaerobic conditions were therefore used. Although rabbit muscle 7-acetylflavo-GAPDH (2d) is too fragile for stopped-flow experiments, we have been able to obtain very interesting results with the bacterial enzyme (Hilvert, Hatanaka and Kaiser, 1987). In the presence of excess NADH, the bleaching of the enzyme-bound flavin cofactor follows biphasic kinetics. Approximately half ( $42 \pm 4\%$ ) of the isoalloxazines are reduced

in a very rapid step, and the remainder at a much lower rate. This behaviour is typical of negatively co-operative enzymes that exhibit half of the sites activity. In this case, binding of the first molecule of NADH may induce a conformational change in the adjacent subunit that turns off its ability to bind another substrate molecule. With a non-specific substrate like PNAH (*N*-propyl-1,4-dihydronicotinamide), on the other hand, reactivity of all of the sites is observed: that is, only a single first-order process is detected, by which all the enzyme-bound flavins in the sample are reduced. The latter result argues against the possibility that our semisynthetic enzyme preparations contain a heterogeneous mixture of chemically distinct binding sites.

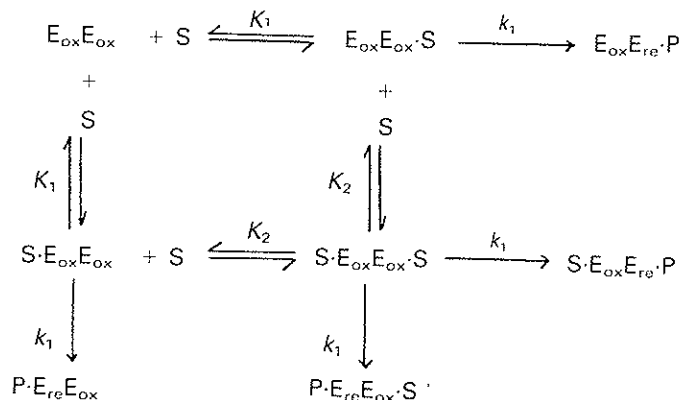
The rate data for the fast-phase reduction of 7-acetylflavo-GAPDH (2d) by NADH follow saturation kinetics. Assuming again, for the sake of analysis, that a simple sequential interaction model is an appropriate description of the behaviour of the multimeric semisynthetic flavoenzyme (*see Figure 5*) (Segel, 1975), equation 3 holds.

$$k_{\text{fast}} = \frac{v}{[E]_{\text{T}}} = \frac{2k_1 \left[ \frac{S}{K_1} + \frac{S^2}{K_1 K_2} \right]}{\left[ 1 + \frac{2S}{K_1} + \frac{S^2}{K_1 K_2} \right]} \approx \frac{k_1 S}{\left[ \frac{K_1}{2} + S \right]} \quad (3)$$

In this equation  $k_1$  is the microscopic first-order rate constant for transfer of a hydride from bound NADH to the flavin attached to Cys-149, and  $K_1$  and  $K_2$  are the respective dissociation constants for the two active sites of the dimer. When  $K_2 \gg K_1$ , equation 3 simplifies and takes the form of the standard Michaelis-Menten equation. The stopped-flow data are fitted quite well by the simplified equation (a double reciprocal plot has a correlation coefficient of 0.98) and give  $k_1 = 1.14 \text{ s}^{-1}$  and  $K_1/2 = 12.4 \mu\text{M}$  (Hilvert, Hatanaka and Kaiser, 1987). Thus, the apparent second-order rate constant for the fast subunit of the enzyme is  $91\,900 \text{ M}^{-1} \text{ s}^{-1}$ . This value is only a little larger than the steady state value of  $k_p/K_s$  determined for NADH with this enzyme, and it compares very favourably with  $k_{\text{cat}}/K_m$  values obtained for naturally occurring flavoenzymes that oxidize NADH. More importantly, the rate of the hydride transfer step is also substantial ( $k_1 = 1.14 \text{ s}^{-1}$ ). As 2d represents only a first-generation catalyst, it may be possible to improve the transition state geometry by rational design. In this way, further increases in the value of  $k_1$  might be achieved, possibly by an order of magnitude or two.

#### ACCEPTORS

Naturally occurring flavoenzymes catalyse the oxidation of NADH with turnover numbers in the range  $0.5 - 35 \text{ s}^{-1}$ . Given a suitable electron acceptor to reoxidize the flavin, the turnover number of bacterial 7-acetylflavo-GAPDH (2d) could therefore approach that of these natural systems (up to  $1 \text{ s}^{-1}$ ). Unfortunately, the electron-accepting dyes that were used successfully



**Figure 5** Scheme for simple sequential interaction model where S and P are NADH and  $NAD^+$ , respectively;  $E_{ox}$  is the oxidized subunit of dimeric 7-acetyl-flavo-GAPDH;  $E_{re}$  is the reduced subunit of the enzyme; and  $E_{ox}\cdot NADH$  and  $E_{re}\cdot NAD^+$  are the various possible Michaelis complexes.

with flavopapain are actually worse oxidants for GAPDH-bound dihydroflavin than molecular oxygen (Hilvert, Hatanaka and Kaiser, 1987). DCIP and MTT are relatively large flat molecules that must bind at the active site in order to be effective. It is possible that they are unable to compete effectively with  $NAD^+/NADH$  for available free sites on the enzyme. Preliminary experiments with ferricyanide, which is substantially smaller than the electron-accepting dyes, indicate that this reagent may be an efficient oxidant for the reduced enzyme (D. Hilvert and Y. Hatanaka, unpublished results). In addition, recent work with cytochrome *c* shows that this macromolecule also slightly enhances the turnover rate of the bacterial dimer in air-saturated buffer (T. Kokubo, unpublished results). In the latter case, it is possible that cytochrome *c* is able to form a complex with the dimeric flavoenzyme that facilitates the rate of electron transfer. This particular oxidant does not enhance the turnover rate of tetrameric 7-acetylflavo-GAPDH from rabbit muscle, however (D. Hilvert, unpublished results).

#### SPECIFICITY

Large rate accelerations are only one aspect of enzyme-catalysed reactions. Equally important is the ability of an enzyme to promote reactions with precise regio- and stereoselective control. We have studied the stereospecificity of hydride transfer from NADH to the bound flavin in 2d using chirally deuterated NADH derivatives (Hilvert and Kaiser, 1985; Hilvert, Hatanaka and Kaiser, 1987). The product ratios for the oxidation of (4R-D)NADH and (4S-D)NADH by 7-acetyl-10-methylisoalloxazine (2c) and the two semi-synthetic enzymes are summarized in *Table 3*. Surprisingly, only the rabbit muscle enzyme shows a substantial preference for transfer of the pro-S hydrogen (i.e. the specificity of the unmodified enzyme). The thermophilic

**Table 3.** Product ratios<sup>a</sup> for the oxidation of selectively deuterated NADH derivatives by 7-acetyl-10-methylisalloxazine (7-AcFl) and by 7-acetyl-flavo-GAPDH from rabbit muscle and *B. stearrowthermophilus* (Hilvert and Kaiser, 1985; Hilvert, Hatanaka and Kaiser, 1987).

Substrate	(4-D)NAD <sup>+</sup> /(4-H)NAD <sup>+</sup>		
	7-AcFl	Muscle	<i>B. stearrowthermophilus</i>
(4R-D)NADH	1.86	13.3	3.35
(4S-D)NADH	10.8	1.17	6.14

<sup>a</sup>The substrates were oxidized using the conditions described in Table 2, and the product ratios determined as described by Arnold *et al.* (1976).

enzyme actually exhibits slight *re*-face stereoselectivity, as do the non-enzymatic model system and flavopapain. These results are consistent with the observation that the rabbit muscle flavoenzyme is active primarily as a tetramer, whereas the bacterial flavoenzyme is dimeric. The geometry of the binding pocket in the tetramer is likely to be very similar to that observed in the crystal structures of the lobster muscle and *B. stearrowthermophilus* enzymes. If the substrate NADH binds in roughly its normal conformation, it would expose only the pro-S hydrogen to the nearby flavin. The fact that absolute *si*-face stereospecificity is not observed for the rabbit muscle 7-acetylflavo-GAPDH indicates that other reaction geometries are also accessible. It is possible that the lack of complete stereochemical control is due to the relative instability of the rabbit muscle protein. The stereochemical experiments are lengthier than the standard steady state assays; it is therefore possible that some dimer (with its slight preference for pro-R hydrogen transfer) develops during the course of the reaction and catalyses a few turnovers before inactivating. The binding pocket of dimeric 7-acetylflavo-GAPDH (2d) is likely to be much more open than that of the native tetramer. In such an expanded active site, the dihydropyridine moiety of the substrate would be expected to have increased freedom to rotate. Such flexibility would, of course, lead to the loss of any stereochemical imperative imposed by the protein. Moreover, the extra rotational freedom could account for the higher rates observed in the case of bacterial 2d. The greater mobility of the substrate and the flavin may facilitate attainment of better overlap between the reactive centres in the transition state at the cost of stereochemical control.

The last feature of 7-acetylflavo-GAPDH (2d) that should be discussed is its substrate specificity. A priori considerations of the binding site of GAPDH had suggested that hydrophobic *N*-alkyl-1,4-dihydronicotinamides would be poor substrates for the semisynthetic enzyme. For both the rabbit muscle and bacterial enzymes this is the case. Although apolar substrates show normal saturation kinetics with both catalysts, the rate accelerations over the analogous non-enzymatic model reactions are negligible. The kinetic parameters for a typical example, the oxidation of *N*-propyl-1,4-dihydronicot-

inamide, are summarized in *Table 2*. The second-order rate constant for oxidation of this substrate by 7-acetyl-10-methyl-isoalloxazine is  $948 \text{ M}^{-1} \text{ s}^{-1}$ . Comparison of this value with  $k_p/K_s$  gives a modest twofold rate acceleration for bacterial 7-acetylflavo-GAPDH and a slight *deceleration* with the rabbit muscle protein. These findings contrast with those obtained with flavopapain in an entirely predictable fashion. By carefully choosing a protein template, it is thus possible to design semisynthetic enzymes with specific substrate preferences.

In principle, even finer substrate selectivity should be attainable than the simple discrimination between polar and apolar dihydronicotinamides. Native GAPDH utilizes  $\text{NAD}^+/\text{NADH}$  exclusively.  $\text{NADP}^+/\text{NADPH}$ , differing from  $\text{NAD}^+/\text{NADH}$  only in its extra phosphate group, is not a substrate for the unmodified enzyme at all. Consistent with this behaviour,  $\text{NADPH}$  oxidation is only weakly catalysed by rabbit muscle 2d (*Table 2*). The apparent bimolecular rate constant ( $57 \text{ M}^{-1} \text{ s}^{-1}$ ) is only 2–3-fold larger than that for the non-enzymatic reaction catalysed by 7-acetyl-10-methylisoalloxazine ( $k_2 = 21.7 \text{ M}^{-1} \text{ s}^{-1}$ ). Thus, the binding template is largely intact for the rabbit muscle semisynthetic flavoenzyme: it exhibits the expected stereoselectivity, as discussed above, and the expected substrate specificity. Quite different results were obtained, however, when we examined the oxidation of  $\text{NADPH}$  catalysed by bacterial 7-acetylflavo-GAPDH (2d). This compound is actually a very good substrate for the thermophilic semisynthetic enzyme, almost as effective as  $\text{NADH}$  itself (*Table 2*). Like  $\text{NADH}$ , its kinetic profile shows apparent negative co-operativity, and at low substrate concentrations the effective enzymatic rate enhancement is almost 1000-fold. This acceleration is comparable to that observed for flavopapain with its best substrate. In addition, reactivity of half of the sites is found when the reduction of the flavin chromophore by an excess of substrate is monitored using stopped-flow techniques.  $\text{NADPH}$  thus appears to be a very specific substrate for the bacterial semisynthetic enzyme. Why is this so? A reasonable answer to this question can be found in the quaternary structure of the catalyst.  $\text{NADPH}$  binding to the native tetramer is normally prevented by unfavourable *intersubunit* contacts across the crystallographic Q-axis (*Figure 4*). The relatively large  $K_s$  value obtained with  $\text{NADPH}$  and the tetrameric rabbit muscle catalyst, for example, is indicative of weak binding. However, bacterial 7-acetylflavo-GAPDH is dimeric. If dissociation of the tetramer were to occur along the Q-axis, as suggested above, the binding pocket for the adenosyl portion of the substrate would become solvent accessible and able to accommodate the steric and electronic demands of the extra phosphate group of  $\text{NADPH}$ . Model building using molecular graphics supports these conclusions (Hilvert, Hatanaka and Kaiser, 1987). Thus, it is possible to interpret both the stereoselectivity and substrate specificity of the bacterial enzyme using a satisfactory structural model.

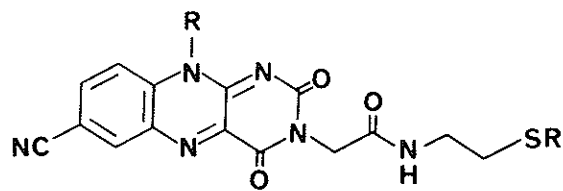
In short, high rates, substrate specificity and, in the case of the muscle enzyme, substantial stereoselectivity, characterize the reactions catalysed by 7-acetylflavo-GAPDH (2d). This work amply demonstrates that GAPDH is a viable and valuable template for the construction of artificial flavoenzymes.



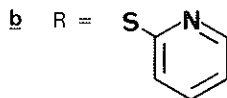
Moreover, GAPDH is an ideal vehicle for the development of second- and third-generation catalysts using rational design strategies. We are confident that even better turnover numbers and rate accelerations can be achieved by optimizing the orientation of the catalytic cofactor within the binding pocket and its mode of attachment to Cys-149. Knowledge of how NADH normally binds to the enzyme will facilitate such a process. Simply shifting the position of the acetyl group through which the flavin is attached to the protein, however, is not sufficient to improve enzymatic activity. Rabbit muscle 6-acetylflavo-GAPDH (4d) and 8-acetylflavo-GAPDH (3d) have also been prepared and subjected to preliminary characterization, but are poor catalysts relative to 7-acetylflavo-GAPDH (Hilvert and Kaiser, 1985; D. Hilvert, unpublished results). We have consequently begun to synthesize smaller redox cofactors, pterin derivatives containing reactive side chains, for incorporation into the active site of the enzyme (Y. Hatanaka and D. Hilvert, unpublished results). Pterins are likely to be easier to position within the binding pocket, and their small size should not perturb the quaternary structure of the template. The latter point seems to be especially important for achieving high stereoselection and substrate specificity in these semisynthetic enzymes. Another viable approach to optimizing catalytic activity involves altering the structure of the binding pocket itself. The availability of multiple naturally occurring structural variants is a particularly attractive feature of the GAPDH template, that may ultimately permit manipulation of the properties of the semisynthetic catalysts based on it in a rational way. GAPDH from yeast, for example, exhibits positive rather than negative cooperativity as seen for the rabbit muscle and *B. stearrowthermophilus* enzymes; GAPDH from the extreme thermophile *Thermus aquaticus*, on the other hand, should be even more stable than the protein from *B. stearrowthermophilus*. It would be very interesting to study the behaviour of the flavoenzymes based on these enzymes, especially their differing stabilities, redox properties and kinetic efficiencies. Ultimately, site-directed mutagenesis using recombinant DNA technology could be employed in combination with the chemical mutation strategy to tailor the ideal catalyst for a given situation.

### Flavohaemoglobin

Recently, we have generated a new type of semisynthetic enzyme, flavohaemoglobin, in which the haem group is maintained in the protein but a flavin molecule is added by covalent modification (Kokubo, Sassa and Kaiser, 1987). It is known that haem proteins such as cytochrome P-450, which carry out redox reactions, function typically by means of single electron transfers in each step (Sato and Omura, 1978). When they operate with the usual biological electron donors such as NADPH, which act by two electron transfers, there is a requirement for an electron transport system. We sought to determine whether haemoglobin could be modified in such a fashion that it might react directly with two electron donors without the intervention of the full electron transport system. Accordingly, we attached an isoalloxazine residue covalently to haemoglobin in the vicinity of the haem with the hope



**5a** R = S-haemoglobin



**Figure 6** Preparation of flavohaemoglobin species from haemoglobin and isoalloxazine residue.

that the flavin moiety could mediate electron transfer obviating the need for the P-450 reductase (NADPH-ferrihemoprotein reductase; EC 1.6.2.4). We found that the flavohaemoglobin species **5a** (*Figure 6*) did indeed serve as a hydroxylase for aniline in the absence of the reductase protein. The chemical modification was carried out by reaction of a derivative of 7-cyanoisoalloxazine with the thiol group of cysteine  $\beta$ -93 which is located next to the histidine ( $\beta$ -92) that is co-ordinated to the haem iron. To prepare the flavohaemoglobin, carbonmonoxyhaemoglobin ( $\text{COHb}^{2+}$ ) was treated with compound **5b** (*Figure 6*) at pH 7 to obtain the covalently modified species  $\text{Fl-COHb}^{2+}$ . Subsequently, ferric flavohaemoglobin ( $\text{FlHb}^{3+}$ ) was prepared by oxidation of  $\text{Fl-COHb}^{2+}$  with potassium ferricyanide.

#### KINETICS

We studied the aniline hydroxylase activity of  $\text{FlHb}^{3+}$  at pH 7.5, varying the aniline concentration at several fixed concentrations of NADPH (Mieyal *et al.*, 1976). We found that the rate of hydroxylation of aniline was dependent on the concentration of both aniline and NADPH, similar to the kinetic behaviour of two-substrate enzyme reactions. We obtained straight lines intersecting at a single point in double reciprocal plots of the rate for various concentrations of NADPH. This demonstrated that NADPH as well as aniline behaves as a substrate. Further analysis of the kinetic data showed that  $k_{\text{cat}} = 0.26 \text{ min}^{-1}$ ,  $K_m(\text{aniline}) = 5.5 \text{ mM}$  and  $K_m(\text{NADPH}) = 0.22 \text{ mM}$ , respectively. The  $k_{\text{cat}}$  values observed for microsomal cytochrome P-450 in the hydroxylation of aniline range from 0.22 to 0.65  $\text{min}^{-1}$ , according to the literature (Lu *et al.*, 1972; Takikawa, Yoshida and Hayaishi, 1983). Clearly,

the catalytic activity of Fl-Hb<sup>3+</sup> as a hydroxylase is comparable to that of microsomal cytochrome P-450.

We found that the apparent  $K_m$  values for aniline observed for Fl-Hb<sup>3+</sup> compared with ferrihaemoglobin, Hb<sup>3+</sup>, assayed in the presence and absence of NADPH-cytochrome P-450 reductase were essentially the same. However, the apparent  $k_{cat}$  for Hb<sup>3+</sup> was increased considerably when we reconstituted that system with the P-450 reductase. Still, the apparent  $k_{cat}$  for Hb<sup>3+</sup> in the reconstituted system was smaller than that seen for Fl-Hb<sup>3+</sup> by itself. Thus, the flavin covalently bound to haemoglobin in Fl-Hb<sup>3+</sup> is able to substitute effectively for the reductase. It appears that electron transfer between the neighbouring prosthetic groups in Fl-Hb<sup>3+</sup> proceeds even more efficiently than that in the combined Hb<sup>3+</sup>-reductase system. In further work we are attempting to determine whether Fl-Hb<sup>3+</sup> acts by a mechanism similar to that of cytochrome P-450, and we are probing further the catalytic versatility of the Fl-Hb<sup>3+</sup> system.

### Conclusion

Some day general rules for designing catalytically active proteins from their constituent amino acids will become available. Nevertheless, such procedures will probably never be easy. The construction of semisynthetic enzymes provides an alternative, and completely general, strategy for catalyst design using existing protein structures as versatile scaffolding on which to mount catalytic functionality. As such, it complements other current approaches to this general problem, including (1) site-directed mutagenesis of proteins using recombinant DNA techniques (Ulmer, 1983); (2) random mutagenesis on micro-organisms and selection for 'improved' enzymes; (3) production of catalytically active antibodies (Hansen, 1987); and (4) the development of low-molecular-weight artificial enzymes based on cyclodextrins or purely synthetic binding cavities (Breslow, 1982).

As the foregoing discussion demonstrates, we have already reached the stage that effective semisynthetic flavoenzymes can be built from a range of templates to have different binding specificities and reactivities. In addition to those protein templates considered explicitly above, preliminary studies of others have been initiated. These include chymotrypsin (EC 3.4.21.1) (Kaiser *et al.*, 1980), subtilisin (EC 3.4.21.14) (D. Lawrence, unpublished results) and lysozyme (EC 3.2.1.17) (Rokita and Kaiser, 1986). Using such proteins we hope to generate a family of flavoenzymes that will yield detailed information about the relationship between protein structure and catalytic activity. Ultimately, such knowledge will be used to construct even more effective second- and third-generation catalysts.

It is conceivable that naturally occurring flavoenzymes (and other cofactor-dependent proteins) may have evolved by an exploratory process much like the one we have employed. At one time, various proteins could have acted as templates for a given prosthetic group, with the best catalysts surviving to the second, third or fourth generation. Coupling such a random search

process with gradual mutagenesis of the template proteins would have permitted optimization of the primitive catalysts for their metabolic roles.

Although the development of semisynthetic enzymes is still in its infancy, we foresee many practical applications of these catalysts. We have modelled primarily the transhydrogenase activity of flavoproteins, but flavoenzymes also carry out many interesting oxygen transfer reactions that are difficult to accomplish using standard chemical methodology. The hydroxylation of phenols and the stereospecific oxidation of thiol ethers to chiral sulphoxides are obvious choices for study with our flavin-dependent semisynthetic enzymes. With flavohaemoglobin, on the other hand, we may be able to mimic some of the very interesting chemistry of the cytochrome P-450 enzymes. The advantage of semisynthetic enzymes over their naturally occurring counterparts derives from their versatility. By simply changing the protein template it is possible to adjust the pH optimum, improve catalyst stability and fine-tune both the substrate specificity and the stereoselectivity of the reaction under consideration. Of course, it will always be necessary to optimize each semisynthetic catalyst for its specific application, but with luck this process will become easier as the general rules that relate protein structure with function are elucidated.

In closing, we should note that the chemical mutation strategy is not restricted to flavin cofactors. Many other prosthetic groups are excellent candidates for use in a semisynthetic enzyme. Pterins have already been mentioned, but other coenzymes, like thiamine, pyridoxal, porphyrins, phthalocyanines, even metal ions, could be incorporated into protein binding sites to give novel, practical catalysts. Exploration of this exciting new frontier in catalyst design enables us to begin to answer some of the questions raised in our opening paragraph and brings us a little closer to our goal of being able to design enzyme-like catalysts for any chemical transformation.

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