Active Site Probes of Flavoproteins

DETERMINATION OF THE SOLVENT ACCESSIBILITY OF THE FLAVIN POSITION 8 FOR A SERIES OF FLAVOPROTEINS*

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The chemical reactivity of 8-chloroflavins and 8-mercaptoflavins has been exploited in order to examine the orientation of protein-bound flavins relative to solvent. The apoprotein form of a series of flavoproteins was prepared and the native flavin was replaced by either 8-Cl-flavin or 8-mercaptoflavin (FAD, FMN, or riboflavin form as was appropriate). The reconstituted proteins were exposed to reagents capable of reacting with the group at position 8. The 8-Cl-proteins were challenged with sodium sulfide and thiophenol, while the 8-mercaptoproteins were faced with iodoacetamide and iodoacetic acid. The kinetics of the ensuing reactions served as a measure of the solvent availability of position 8 for the protein-bound flavin. These studies indicated that position 8 of flavin bound to melittate hydroxylase, p-amino acid oxidase, old yellow enzyme, p-OH-benzoate hydroxylase, and flavodoxin is accessible to solvent, while position 8 on l-lactate oxidase, glucose oxidase, putrescine oxidase, and riboflavin-binding protein appears to be inaccessible. For luciferase, p-lactate dehydrogenase, and xanthine oxidase, the data suggest that position 8 is exposed but the results are inconclusive. The effect of ligand binding on the accessibility of position 8 was also studied. NADPH binding to 8-mercapto old yellow enzyme and benzoate binding to 8-Cl-p-amino acid oxidase results in complete blockage of previously available position 8. On the other hand, p-OH-benzoate hydroxylase and melittate hydroxylase bind their respective substrates (p-OH-benzoate and melittate) without significantly altering the reactivity of position 8.

A wide variety of biochemical reactions depend on flavoproteins for catalysis (1). The catalytically active moiety (the flavin isoalloxazine ring system) is the same in all flavoproteins. It has therefore been suggested that the specificity of these reactions stems from unique interactions between the flavin and the protein (1). These interactions can be envisioned as directing the versatile oxidation-reduction properties of the flavin to accommodate the varying demands put upon it by each different reaction. That the flavin environment is perturbed on binding to different proteins has long been apparent in the unique UV-visible absorbance spectra of different flavoproteins and in the varying capacity of different flavoproteins to quench the intrinsic fluorescence of free flavin (2, 3). Early studies on the effect of solvent on the free flavin absorbance spectrum indicated that, in general, the protonic environment was important in refining the shape and position of the absorbance peaks (3-5). Fluorescence quenching has been attributed to interactions with aromatic amino acid side chains (6).

Subsequent studies with a variety of techniques have provided support for the thesis that the protein affords a unique environment for the flavin. Circular dichroism studies on flavodoxin (7), Shethna flavoprotein (7), l-amino acid oxidase (7), glucose oxidase (7), and acyl-CoA dehydrogenase (8) suggest that the flavin environment in the oxidases is distinctly different from that in flavoproteins which do not react rapidly with oxygen. Resonance Raman and coherent anti-Stokes Raman spectroscopic investigations have shown clear, protein-dependent changes in the flavin vibrational structure (9-11). 13C-NMR spectra of riboflavin bound to riboflavin-binding protein show a perturbation of the signal from C(4) carbon suggesting an interaction between the protein and the flavin (12). Proton relaxation measurements have shown the central pyrazine subnucleus of the semiquinone forms of flavodoxin (13) and methanol oxidase (14) to be accessible to solvent protons. X-ray crystal structures of flavodoxin (15) and glutathione reductase (16) show significant differences in the flavin environment of these two proteins.

Recent efforts to define flavin-protein interactions have capitalized on differences in the chemical reactivity of the protein-bound flavin. Studies with native flavoproteins on sulfite reactivity (17), flavin semiquinone stabilization (17, 18), and oxygen reactivity (17) have led to the suggestion that flavoprotein oxidases all have an essential hydrogen bond in the N(1)-(C(2a)) locus. These and similar studies have suggested that flavoprotein transhydrogenases and hydroxylases require a hydrogen bond at the N(5) position of the flavin (cf. Ref. 1 for a recent review).

With the increasing availability of modified flavins, it has become possible to use several of these as versatile probes with which to examine the influence of the protein on the flavin. The preceding papers in this series on modified flavins have dealt with the use of 1) 8-Cl-flavin as an active site-directed thiol reagent (19), and 2) 8-mercaptoflavin anion as a sensitive monitor of the dipole environment of the active site (20). The results of these studies were consistent with the hydrogen-bonding assignments suggested by the chemical reactivity of protein-bound flavin.

In addition, modified flavins can be used to investigate the solvent accessibility of the protein-bound flavin (20). This approach exploits the chemical reactivity of the group modifying the flavin. In the following work, we have used 8-Cl- and 8-mercapto-modified flavins to probe the solvent accessibility of position 8 for a series of flavoproteins. Both of these

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Dedicated to Professor A. Rossi Fanelli on the occasion of his seventy-fifth birthday.
modifying groups are reactive. The chloride of 8-Cl-flavin readily undergoes nucleophilic substitution by thiolates, including sulfide (19, 21), while 8-mercaptoflavins react readily with iodoacetamide and iodoacetic acid. In both cases, there is a large shift in the UV-visible absorbance spectrum of the flavin, which can be used to follow the reaction (Fig. 2).

Strictly speaking, we are examining the accessibility of position 8 to solvent-borne reagents. However, since the reagents we have employed are larger than solvent, accessibility of position 8 to the reagent implies access to solvent also. On the other hand, inaccessibility of position 8 to the reagent could be the result of steric hindrance which might not apply to the smaller solvent molecules.

With this approach, we have established that position 8 of the flavin in the following flavoproteins is available to solvent: melilolate hydroxylase, p-amino acid oxidase, old yellow enzyme, p-OH-benzoate hydroxylase, and flavodoxin. Further, it appears that in luciferase, D-lactate dehydrogenase, and xanthine oxidase, position 8 of the flavin may also be accessible to solvent. The 8 positions in l-lactate oxidase, glucose oxidase, putrescine oxidase, and riboflavin-binding protein appear to be inaccessible to solvent. For several of these flavoproteins, we have examined the effect of ligand binding on the accessibility of position 8. Our probes allow us to specifically detect changes which affect the environment of position 8. When benzoate is bound to 8-Cl-D-amino acid oxidase or when NADPH is bound to 8-Cl-p-amino acid oxidase or when 8-mercapto old yellow enzyme, previously available position 8 becomes totally blocked. On the other hand, binding melilolate to melilolate hydroxylase (8-Cl or 8-mercapto forms) or p-OH-benzoate to p-OH-benzoate hydroxylase (8-Cl or 8-mercapto forms) does not appear to cause major structural changes in the vicinity of position 8.

**MATERIALS AND METHODS**

Enzymes and the corresponding apoenzymes used in this study were prepared as previously described. Flavodoxin from *Megasphaera elsdenii* (22, 23), l-lactate oxidase from *Myco bacterium smegmatis* (24, 25), p-amino acid oxidase from pig kidney (26, 27), glucose oxidase from *Aspergillus niger* (28, 29), melilotate hydroxylase from *Pseudomonas* sp. (30), old yellow enzyme from brewers' yeast (31, 32), riboflavin-binding protein from hen egg white (33, 34), and p-OH-benzoate hydroxylase from *Achromobacter* sp. (35, 36). The following enzymes were provided as generous gifts: xanthine oxidase from milk, by Dr. R. Hille; p-lactate dehydrogenase from *M. elsdenii*, by Dr. F. F. Morpeth (both of whom are at the University of Michigan); luciferase from *Benechea harveyi* (strain M-17), by Dr. T. Baldwin, University of Illinois; and putrescine oxidase from *Micrococcus rubens*, by Dr. R. DeSa, University of Georgia.

Modified FAD was prepared from the appropriate riboflavin using the FAD synthetase complex, partially purified from *Brevibacterium ammoniagenes*, as described by Spencer et al. (37). Modified FMN was prepared from the appropriate FAD by treatment with *Naja naja* venom (Sigma). The modified riboflavin were generous gifts: 8-Cl-riboflavin from Dr. J. P. Lambooy, University of Maryland; 6-OH-riboflavin from Dr. P. Hemmerich, University of Konstanz, West Germany; and 1-deazariboflavin from Merck, Sharp and Dohme Research Laboratory, Rahway, NJ. 8-Mercaptoflavins were prepared from the appropriate 8-Cl-flavin by reaction with Na2S as described by Moore et al. (21). 2-OH-Phenyl propionate (mellitate) was prepared from dihydrocumarin (Aldrich) by base hydrolysis (30). Iodoacetamide, iodoacetic acid, and NADPH (type II) were from Sigma; thiophenol (mercaptobenzene) was from Aldrich and sodium sulfide was from Mallinckrodt. All other chemicals were of reagent quality.

All reactions were carried out at 25 °C in 0.1 M potassium phosphate buffer, pH 7.3, except those involving luciferase. The luciferase studies were performed at 4 °C (in the above buffer) in order to maximize the binding of flavin.

All absorbance measurements were made with temperature-controlled double beam spectrophotometers, Cary 17, 118, or 219. The cell compartments were flushed with dry air to prevent fogging during the 4 °C experiments.

The sodium sulfide reactions were conducted under a single fixed set of conditions in order to facilitate comparison of the resulting complex spectral changes. To 1 ml of buffer, containing the appropriate flavoprotein (8-Cl derivative), was added 10 μl of a 1 M solution of sodium sulfide. The pH of the reaction consistently rose to 7.8.
Progress of the reaction was followed by the absorbance rise in the region between 550 and 600 nm (Fig. 2B). The peak position varied widely throughout this region depending upon the protein (20). Thiophenol reactions were initiated by adding 2 to 50 μl of thiophenol (diluted 1:1000 in methanol) to 0.8 ml of buffer containing the appropriate flavoprotein (8-Cl derivative) and 1.25 mM EDTA. The cuvette was stopped but not deaerated. EDTA was included to reduce trace metal catalysis of thiophenol oxidation. At the end of prolonged reactions, i.e. greater than 1 h, the presence of thiophenol in the cuvette was qualitatively affirmed by its odor. The pronounced rise in absorbance around 480 nm upon converting 8-Cl-flavin to 8-thiophenylflavin (Fig. 2B) afforded a convenient measure of the reaction progress.

In control experiments, the addition of 50 μl of methanol to the reaction (1.3% v/v) resulted in a slight decrease (10 to 40%) in the observed rate both for the free and protein-bound 8-Cl-flavin reactions. The effect was least on the rate with free 8-Cl-flavin. Analysis of the kinetics (below) generally disregards this methanol effect. However, 8-Cl-melilotate hydroxylase was exceptionally sensitive to the added methanol. By way of compromise, reactions with 8-Cl-melilotate hydroxylase were performed at a constant methanol concentration. The resultant rates, although depressed, were still markedly faster than those found for the comparable free flavin reaction, thus the interpretation is not compromised (see below).

Reactions with either iodocetamide or iodicetic acid were initiated by adding an aliquot of one of these reagents (1 μl stock) to 0.8 ml of buffer containing the appropriate flavoprotein (8-mercaptopropionate derivative). The iodicetic acid was neutralized to pH 7 prior to use. The resulting spectral changes were quite large (Fig. 2A) and were monitored as the loss of 8-mercaptopropionate absorbance (530 to 600 nm) or the rise of 8-SR-flavin absorbance (approximately 480 nm). Rates at both wavelengths were the same.

Not all of the flavoproteins studied were reactive toward the reagents. However, those that reacted did so in a pseudo-first order manner (with thiophenol, iodocetamide, and iodicetic acid). In an effort to simplify the presentation of results, we have qualitatively divided the following experiments into four categories, based on the observed kinetics.

Case 1 includes all those flavoproteins for which little or no reaction could be observed after 6 h in the presence of the reagent. These flavoproteins are tentatively considered to have inaccessible flavins, although other interpretations are possible and some of these are discussed below.

Case 2 includes all of the reactions which showed a simple second order dependence of the observed rate (kobs) on reagent concentration, i.e. a plot of kobs versus reagent concentration was linear and passed through zero. This case was considered to involve a one-step reaction between reagent and flavin (flavoprotein):

\[
EFl + [R]\rightarrow EFlR
\]

**SCHEME 1**

EFl is the flavoprotein, R is the reagent, EFlR is the reaction product, and k is the second order rate constant. For those reactions in which both the decay of substrate and the appearance of product could be measured independently, the second order rate constant was found to be the same for either species. In practice, the second order rate constant measured for the flavoprotein reaction was larger than that for the comparable free flavin reaction. This observation can be rationalized only if the protein-bound flavin can react directly with the reagent. Thus, position 8 of the protein-bound flavin must be accessible to solvent.

Case 3 includes all of those reactions which were rapid (relative to the free flavin reaction) but for which the dependence of kobs on reagent concentration was not simple second order. In this instance, we have chosen to compare directly the apparent first order rate for the flavoprotein reaction with that for the free flavin reaction under equivalent conditions. We have found that the flavoprotein reaction was invariably faster than the corresponding free flavin reaction, leading us to conclude that the protein-bound flavin is reactive and that position 8 is exposed to solvent.

When the dependence of kobs on reagent concentration appears to be parabolic, as was often the case, it is tempting to linearize the data with a double reciprocal analysis (38). However, the double reciprocal analysis implies that an intermediate complex, such as a Michaelis complex, occurs prior to product formation. In view of the reagents used in this work, we considered a mechanism involving a Michaelis complex unlikely (although not impossible). Thus, we have chosen not to analyze our data in the double reciprocal fashion and have instead employed the strategy described above.

Case 4 includes those reactions which were slow (relative to the free flavin reaction) but for which a rate could be measured. While the observed slow reaction could obviously be due to impaired access of the reagent to the enzyme-bound flavin, it appears that in all of the examples which we have examined, the observed reaction involves dissociation of the flavin from the protein followed by reaction of the flavin with reagent free in solution. The possibility of subsequent rebinding of the reaction product to the apoprotein is not pertinent to the analysis. This situation is described by:

\[
EFl + [R] \rightarrow E + FI + [R] + \text{product}
\]

**SCHEME 2**

Where EFl is the flavoprotein, E is the apoprotein, FI is the free flavin, R is the reagent, and FIR is the reaction product. An expression for the observed rate of disappearance of substrate (kobs) can be derived using the steady state approach, if the concentrations of apoprotein (E) and reagent (R) are assumed to remain constant. In practice, these assumptions can be justified by using 5- to 10-fold more apoprotein and reagent than flavin. The expression for kobs, thus obtained:

\[
k_{obs} = \frac{[R]k_{k3}}{[E]k_{p2} + [R]k_{k3}}
\]

**Equation 1**

Rate constants k1 and k2 are the dissociation and association rate constants for the interaction of flavin with apoprotein. Rate constant k3 is the second order constant for the reaction of flavin and reagent free in solution. Concentrations R and E are experimentally defined. Whenever possible, kobs was calculated according to Equation 1 from independently determined values for k1, k2, and k3. The calculated value for kobs invariably matched the measured kobs within a factor of 2, which was considered to be confirmation of the dissociative mechanism. Proteins exhibiting this type of behavior are tentatively considered to have solvent-inaccessible 8 positions.

**RESULTS AND DISCUSSION**

**Reaction of 8-Cl-Flavin with Sulfide**

The small sulfide molecule would seem at first glance to be an ideal tool for the purpose of probing a protein’s structure. However, the kinetics of the reaction between 8-Cl-flavin and sulfide are complex (both with the free and the protein-bound flavin). Typically, there is an initial lag followed by an acceleration to a logarithmic rate followed in turn by a slow trailing off to completion (21). Although the reaction rate varies with pH, there is no simplification of the kinetics on changing pH from 5 to 9. Nevertheless, a qualitative comparison of reaction rates can be obtained by using the logarithmic middle phase of the reaction. The sulfide reaction was examined under a single fixed set of conditions, see “Materials and Methods,” with several enzymes and the results are shown in Table I. It is clear that the 8-Cl groups of 8-Cl-melilolate hydroxylase, 8-Cl-d-amino acid oxidase, 8-Cl-old yellow enzyme, and 8-Cl-luciferase react with sulfide faster than does the 8-Cl group of the free flavin (Fig. 3, A and B). This strongly suggests that position 8 of these enzymes is accessible to solvent. For melilolate hydroxylase, old yellow enzyme, and d-amino acid oxidase, this conclusion is consistent with the results obtained upon reaction with other reagents (see below). However, luciferase (either 8-Cl or 8-mercaptopropionate forms) is unreactive with sulfide, and the reaction occurs with a lag. In view of the complexity of the sulfide reaction, the solvent accessibility of position 8 in the case of luciferase must be considered tentatively established.

It is of interest to note that the unreactive reagents were all larger than sulfide, which appeared to have access to position 8. It should be noted that the luciferase studies were done at 4°C and at a slightly higher pH (8.1). Although interesting, the sulfide reaction was too complex
behave reaction of thiophenol with 8-C1-flavins and 8-C1-flavoproteins listed in Table I, one of four qualitatively different situations occurred (see "Materials and Methods").

Table I
Rates for the reaction of 8-C1-flavins and 8-C1-flavoproteins with NaZS and thiophenol

| Enzymes                  | Reaction rates |
|-------------------------|----------------|
|                         | NaZS           | Thiophenol   |
|                         | Unliganded     | Liganded*    |
|                         |                | Thiophenol² |
| 8-Cl-FAD                | 0.034 min⁻¹    | 3.2 x 10⁵ M⁻¹ min⁻¹ (1.84 min⁻¹) |
| 8-Cl-FMN                | 0.064 min⁻¹    | 3.2 x 10⁵ M⁻¹ min⁻¹ (1.84 min⁻¹) |
| 8-Cl-FMN L-lactate oxidase |              |              |
| 8-Cl-FAD d-amino acid oxidase | 0.06 min⁻¹ | 4.6 x 10⁴ M⁻¹ min⁻¹ (2.65 min⁻¹) |
| 8-Cl-FAD glucose oxidase | t½ > 1 day     | (No reaction)² |
| 8-Cl-FAD putrescine oxidase |            |              |
| 8-Cl-FAD mellitate hydroxylase | 1.2 min⁻¹ | (No reaction)² |
| 8-Cl-FAD p-OH-benzoate hydroxylase | Equal to 8-Cl-FAD² |              |
| 8-Cl-FMN old yellow enzyme | Very fast²   |              |
| 8-Cl-FMN flavodoxin     |                |              |
| 8-Cl-FMN luciferase     | 0.12 min⁻¹     | (No reaction)² |
| 8-Cl-FAD xanthine oxidase |            | (No reaction)² |
| 8-Cl-FAD d-lactate dehydrogenase | No reaction | (No reaction)² |
| 8-Cl-Rf riboflavin-binding protein | No reaction | (No reaction)² |

* Saturating concentrations of ligand were used: benzoate with 8-Cl-d-amino acid oxidase; mellitate with 8-Cl-mellitate hydroxylase; and p-OH-benzoate with 8-Cl-p-OH-benzoate hydroxylase.

² Reactions were monitored for 12 h.

Numbers in parentheses indicate rates measured in the presence of 575 μM thiophenol.

Pseudo-first order rates were obtained upon reaction with the concentrations of thiophenol indicated in parentheses.

Reactions were performed with 12.5 mM sulfide at pH 7.8.

When an excess of thiophenol was added to a solution of 8-C1-flavin, for purposes of comparison.

Reaction carried out at pH 8.5, 25 °C; the kobs was the same for 8-Cl-FAD p-OH-benzoate hydroxylase and 8-Cl-FAD (kobs = 0.26 min⁻¹ at 5 mM sulfide).

Reaction carried out at pH 8.5, 20 °C, and was complete within 3 min with 10 mM sulfide.

Reaction followed for 5 h at 4 °C.

Rf denotes riboflavin.

Fig. 3. Time course traces for NaZS reacting with various flavins and flavoproteins. A, conditions: 0.1 M KP, pH 7.8 (final measured pH), 25 °C. The reaction was followed, at the wavelength indicated in parentheses, after the addition of 10 mM NaZS to the sample: 137 μM 8-Cl-FAD d-amino acid oxidase (---, 600 nm); 16.0 μM 8-Cl-FAD mellitate hydroxylase (-----, 565 nm) and 20.5 μM 8-Cl-FAD (---, 530 nm). B, conditions: 0.1 M KP, pH 8.1 (final measured pH), 4 °C. Reaction was initiated by adding 10 mM NaZS to the sample: 7.9 μM 8-Cl-FMN luciferase (-----, 590 nm) and 8.4 μM, 8-Cl-FMN (-----, 535 nm).

Case 1, 8-Cl-Glucose Oxidase, 8-Cl-Xanthine Oxidase, 8-Cl-Luciferase, 8-Cl-L-Lactate Oxidase, and 8-Cl-Putrescine Oxidase—These enzymes did not show any tendency to react with thiophenol. From these negative results, we can only offer the possibility that these enzymes might protect position 8 from solvent. However, as will be seen with 8-Cl-flavodoxins, other possibilities are available to explain lack of reaction.

Case 2, 8-Cl-d-amino acid oxidase, 8-Cl Old Yellow Enzyme, and 8-Cl-p-OH-Benzoate Hydroxylase—For these enzymes, a simple second order dependence on thiophenol concentration was observed (Fig. 4). In each case, the second order rate constant was larger than that found for 8-Cl-flavin free in solution. Thus, it follows that position 8 of all three of these proteins must be available to solvent. With regard to p-OH-benzoate hydroxylase, this finding is nicely consistent
riboflavin with excess nonfluorescent 1-deazariboflavin. Ap-
protein was determined by competitively displacing the 8-C1-
appearance of free 8-C1-riboflavin was determined by its char-
determined flavin association and dissociation rates as de-
undertaken.

The rate of fluorescence characteristic fluorescence, which is quenched upon association with riboflavin-binding protein (39). The rate of fluorescence increase (8.4 min⁻¹) was independent of the initial 1-deazari-
fluorimetrically determined) for 8-C1-riboflavin concentration, strongly arguing that this rate was a true measure of 8-C1-riboflavin dissociation from 8-C1-riboflavin-binding protein. The association rate constant (k₂ = 7.0 × 10⁹ M⁻¹ min⁻¹) was calculated from the measured dissociation rate (k₁) and the dissociation constant for 8-C1-riboflavin-binding protein (Kd = 1.2 × 10⁻⁴ M). The dissociation constant was determined from an equilibrium titration, where the loss of 8-C1-riboflavin fluorescence was used to monitor complex formation. The free solution rate constant (k₃) for the reaction of thiophenol with free 8-C1-riboflavin was assumed to be the same as that for 8-C1-FAD and 8-C1-FMN (3.2 × 10⁹ M⁻¹ min⁻¹). The reaction reported in Table I was performed with 575 μM thiophenol (R) in the presence of 6.4 μM uncomplexed apo-riboflavin-binding protein (E). The observed rate was 0.0035 min⁻¹ (Table I). Using the above parameters, the calculated value for k₃ was also 0.0035 min⁻¹. Thus, it can be argued that 8-C1-riboflavin bound to riboflavin-binding protein does not react with thiophenol. This finding is consistent with earlier studies on riboflavin-binding protein which have concluded that the benzene end of the isoalloxa-

The association rate constant (k₂) for reaction of thiophenol with 8-C1-flavodoxin is sufficiently slow that it could be directly measured. In a fluorimeter cell, equimolar concentrations of apoflavodoxin and 8-C1-FMN were mixed and the rate of disappearance of the flavin fluorescence was determined. A plot of inverse fluorescence change versus time was linear for greater than 95% of the reaction, giving a second order rate (k₃ = 2.8 × 10⁶ M⁻¹ min⁻¹). This association rate was combined with the equilibrium dissociation constant (about 10⁻⁹ M, fluorimetrically determined) for 8-C1-flavodoxin to give a calculated dissociation rate (k₁ = 2.8 × 10⁻³ min⁻¹). The free solution rate constant (k₃), for reaction of 8-C1-FMN with thiophenol is 3.2 × 10⁻³ M⁻¹ min⁻¹ (see Table I). These parameters were used to calculate the rate expected for the reaction of 1.15 mM thiophenol (R) with 8-C1-flavo-
doxin in the presence of 42 μM free apoflavodoxin (E). It should be noted that twice as much thiophenol was used in this reaction as was used in the 8-C1 flavodoxin reaction reported in Table I. The calculated rate (0.0018 min⁻¹) was in reasonable agreement with the observed rate (0.0041 min⁻¹).

Thus, thiophenol does not appear to react with flavodoxin-bound 8-C1-FMN, suggesting that position 8 is inaccessible to solvent. This finding is perplexing in view of x-ray crystallography studies which show position 8 of the flavin exposed to solvent (15). We speculated that either the thiophenol molecule was too large to gain access to position 8 or that the bulky tetrahedral transition state at C(8) required for an Sₒ₂-type nucleophilic displacement reaction (the anticipated mechanism for this reaction (42)) could not be supported by flavodoxin. In an effort to circumvent these possible problems, we turned to the reaction of 8-mercaptoflavodoxin with the smaller, electrophilic reagents: iodoacetic acid and iodoacetamide.

**Reaction of 8-Mercaptoflavoxin with Iodoacetamide and Iodoacetic Acid**

Using 8-mercaptoflavin as a probe of solvent accessibility at position 8 presents two major problems. First, some proteins tend to stabilize a quinonoid form of the 8-mercaptoflavin, in which the sulfur is doubly bonded to the benzene moiety and the negative charge resides largely in the vicinity of N(1)-C(2α) (20). The decreased nucleophilicity expected of this form would result in much lower reactivity toward iodoacetamide and iodoacetic acid. Thus, it is difficult to obtain information about the availability of position 8 from these reactions. Second, iodoacetamide and iodoacetic acid are gen-

![Fig. 4](left). Plots of apparent rate versus thiophenol concentration for the reaction of 8-C1-Flavodoxin with 8-C1-FAD and various 8-C1-flavoproteins. Conditions: 0.1 M KP, pH 7.3, plus 1.25 mM EDTA, 25 °C. Solid circles, 8-C1-FAD d-
amino acid oxidase; open circles, 8-C1-FAD p-OH-benzoate hydroxylase; and open squares, 8-C1-FAD-8-C1-

![Fig. 5](right). Plot of apparent rate versus thiophenol concentration for the reaction of 8-C1-Melilotate Hydroxylase with 8-C1-Melilolate Hydroxylase. Conditions: same as in Fig. 4, in addition each assay contained 1.3% methanol (v/v).
eral thiol reagents which can in principle react with protein thiols as fast as or faster than they can react with the protein-bound 8-mercaptopflavin. Protein modification of this sort would complicate the interpretation of the flavin reaction, in that the native protein structure might be perturbed. Nevertheless, the 8-mercaptopflavin probe has provided substantial information on the solvent accessibility of position 8 for several flavoproteins. We used iodoacetic acid as well as iodoacetamide in order to explore the potential effect of a negatively charged reagent on the reaction kinetics.

When 8-mercaptop-FAD was reacted with an excess of iodoacetamide (see "Materials and Methods"), an apparent first order reaction occurred. Plotting the observed rate versus the iodoacetamide concentration gave a straight line passing through the origin (Fig. 6). A second order rate constant (k = 46.3 M⁻¹ min⁻¹) was calculated from these data. The reaction of iodoacetamide with 8-mercaptop-FMN was also examined, simple second order reactions were also found (Table II). The flavoproteins examined could be qualitatively grouped as before.

Case 1. 8-Mercapto-1-lactate Oxidase, 8-Mercapto-p-amino Acid Oxidase, 8-Mercapto glucose Oxidase, and 8-Mercapto putrescine Oxidase—All these enzymes possess the blue spectrum of the quinonoid mesomer (29). The 8-mercaptop group of the flavins on these enzymes was found to be unreactive toward iodoacetamide. As was mentioned above, this result was expected from the chemistry of the thiolate-iodoacetamide reaction. No interpretation regarding the availability of position 8 can be made. No attempt was made to react iodoacetic acid with these proteins.

Case 2. 8-Mercaptoflavodoxin, 8-Mercapto-p-OH-benzoate Hydroxylase, and 8-Mercapto Old Yellow Enzyme—All these enzymes reacted with iodoacetamide in a simple, second order fashion (see Fig. 6). 8-Mercapto-p-OH-benzoate hydroxylase and 8-mecapto old yellow enzyme both reacted at a rate faster than free flavin (Table II). The results for these proteins confirm the findings with 8-chloroflavins and argue that position 8 of both is exposed to solvent.

The reaction of iodoacetic acid with 8-mercaptop-p-OH-benzoate hydroxylase was faster than that with 8-mercaptop-FAD, thereby neatly complementing the iodoacetamide results. But with 8-mercaptop old yellow enzyme, there was a nonlinear dependence of kobs on iodoacetamide concentration (Case 3). However, at 1.25 mM iodoacetic acid, the pseudo-first order rate with 8-mercaptop old yellow enzyme (2.0 min⁻¹) was faster than that with 8-mercaptop-FMN (0.09 min⁻¹). Thus, the iodoacetic acid reaction with old yellow enzyme also complements the iodoacetamide findings.

The reaction of iodoacetamide with 8-mecaptoflavodoxin (k = 49 M⁻¹ min⁻¹) occurred at a second order rate equivalent to that found for free 8-mercaptop-FMN (k = 46 M⁻¹ min⁻¹). In the comparable reaction with iodoacetic acid, 8-mecaptoflavodoxin was 2.5-fold slower than free 8-mercaptop-FMN. These relatively large rate constants suggest that position 8 of flavodoxin is available to solvent. However, if the dissociation of 8-mercaptop-FMN from 8-mecaptoflavodoxin was sufficiently fast, then the observed second order dependence could reflect reactions occurring with free 8-mercaptop-FMN. To clarify this point, a value for the observed rate (kobs) was calculated using Equation 1 (see "Materials and Methods") and compared to the measured value for kobs obtained upon reacting 19.6 mM iodoacetamide (R) with 8-mecaptoflavodoxin in the presence of 29 μM free apoflavodoxin (E). The calculated value (kobs, = 0.014 min⁻¹) was 80-fold slower than the observed value (kobs = 1.17 min⁻¹), thus eliminating the dissociative mechanism from consideration. The values of the rate constants used in

**Fig. 6.** Plots of apparent rate versus iodoacetamide concentration for the simple, second order reaction of iodoacetamide with 8-mercaptop-FAD and various 8-mecaptoflavodoxins. Conditions: 0.1 M KPi, pH 7.3, 25 °C. , 8-mercaptop-FAD p-OH-benzoate hydroxylase; , 8-mercaptop-FMN old yellow enzyme; , 8-mercaptop-FMN flavodoxin; and , 8-mercaptop-FAD. Reaction was initiated by adding iodoacetamide from a 1 mM stock solution.

**TABLE II**

**Rates for the reaction of 8-mecaptoflavins and 8-mercaptopflavodoxins with iodoacetamide and iodoacetic acid**

All reactions were performed in 0.1 M KP, buffer, pH 7.3, 25 °C, unless otherwise indicated.

| Enzymes                                | Reaction rates<sup>a</sup> | Iodoacetamide | Iodoacetic acid | Ligand<sup>b</sup> |
|----------------------------------------|-----------------------------|---------------|-----------------|--------------------|
| 8-Mercapto-FAD                         |                             |               |                 |                    |
| 8-Mercapto-FMN                         |                             |               |                 |                    |
| 8-Mercapto-FAd 1-lactate oxidase       |                             |               |                 |                    |
| 8-Mercapto-FAD p-amino acid oxidase    |                             |               |                 |                    |
| 8-Mercapto-FAD glucose oxidase         |                             |               |                 |                    |
| 8-Mercapto-FAD putrescine oxidase      |                             |               |                 |                    |
| 8-Mercapto-FAD melilolate hydroxylase  |                             |               |                 |                    |
| 8-Mercapto-FAD p-OH-benzoate hydroxylase |                |               |                 |                    |
| 8-Mercapto-FMN old yellow enzyme       |                             |               |                 |                    |
| 8-Mercapto-FMN flavodoxin              |                             |               |                 |                    |
| 8-Mercapto-FMN luciferase              |                             |               |                 |                    |
| 8-Mercapto-FAD xanthine oxidase        |                             |               |                 |                    |
| 8-Mercapto-FAD dehydrogenase           |                             |               |                 |                    |

<sup>a</sup> The numbers in parentheses are apparent reaction rates in the presence of 12.5 mM reactant.

<sup>b</sup> Saturating concentrations of ligand were used: benzoate with 8-mercaptop; p-OH-benzoate with 8-mecapto melilolate hydroxylase; p-OH-benzoate with 8-mercaptop p-OH-benzoate hydroxylase; and NADPH with 8-mercaptop old yellow enzyme.

<sup>c</sup> Reactions were performed in 0.1 M KP, buffer, pH 7.3, 4 °C.

<sup>d</sup> Not determined.

<sup>e</sup> Plot of kobs versus reactant concentration was nonlinear.
calculating $k_{obs}$ were independently measured. The association rate ($k_2 = 3.1 \times 10^7 \text{ M}^{-1} \text{ min}^{-1}$) was obtained by following the absorbance change which occurred on mixing 11.2 \text{ M} \text{ apo-flavodoxin with 1.14 M 8-mercaptop-FMN. The dissociation rate (}$k_1 = 0.015 \text{ min}^{-1}$) was obtained by following the rate of appearance of 8-mercapto old yellow enzyme after mixing 11.1 \text{ M} \text{ apo-old yellow enzyme with 1.4 M 8-mercaptopflavodoxin. This rate was independent of the initial apo-old yellow enzyme concentration, supporting our assignment of it as the dissociation rate. This determination was made possible because of the different spectral properties of 8-mercaptopflavodoxin and 8-mercapto old yellow enzyme (20); a wavelength of 600 nm was chosen for analysis.}

Since it was conceivable that the general thiol reagents used in this study could have reacted with the flavodoxin thiols, we measured directly the rate at which iodoacetamide reacted with the protein thiols of flavodoxin. Flavodoxin from *M. elsdenii* has been found to contain 2 cysteine residues. In apoflavodoxin, both react readily with thiols. But when the flavin is present, thiol reactivity is sluggish. Further, when the thiols are modified, the flavin will not bind (43). We used the gain in FMN fluorescence upon its release from modified flavodoxin as an indication of cysteine modification. When native flavodoxin was incubated with 12.5 mM iodoacetamide (5 to 38 mM iodoacetamide was used in the 8-mercaptopflavodoxin reactions), a first order increase in fluorescence occurred at 0.095 min$^{-1}$. This rate was insignificant in comparison with the rates at which iodoacetamide reacted with the flavin thiol of 8-mercaptopflavodoxin (0.26 to 1.85 min$^{-1}$). Similar experiments were performed using 8-mercaptopflavodoxin and 8-SCH$_2$CONH$_2$ flavodoxin.\(^1\) The appearance of free flavin fluorescence was even slower in these cases ($t_1/2$ of several days). These findings argue against the modification of the protein by iodoacetamide during the course of the 8-mercaptopflavodoxin reactions.

Thus, position 8 of flavodoxin appears to be available to solvent, in agreement with the x-ray crystallography data (15). That thiophenol (as well as sulfide) did not react with 8-CI-flavodoxin must be attributed to factors other than inaccessibility of position 8 to solvent, as was suggested at the end of the section on 8-CI-flavin reactions.

Case 3, 8-Mercapto-4-methyl-lactate Dehydrogenase, 8-Mercapto-4-lactate Dehydrogenase, and 8-Mercaptoxanthine Oxidase—These enzymes all showed nonlinear dependence of $k_{obs}$ on reagent concentration in their reactions with iodoacetamide (see Fig. 7). Although this behavior could be interpreted as binding of iodoacetamide to the enzyme prior to covalent modification of the flavin, we feel that such a complex is unlikely (see "Materials and Methods"). The apparent first order rate ($k_{obs} = 0.21 \text{ min}^{-1}$, at 12.5 mM iodoacetamide) for 8-mercaptoplactate hydroxylase (which binds and is available to solvent) was 50% slower than that found with 8-mercaptop-FAO ($k_{obs} = 0.46 \text{ min}^{-1}$). However, we do not believe that this lower rate indicates inaccessibility of position 8 to solvent. The preceding experiments involving reaction of the 8-chloro-FAO enzyme with both thiophenol and sulfide indicated that position 8 of melittate hydroxylase was available to solvent. In addition, the reaction of 8-mercaptoplactate hydroxylase with iodoacetate occurred at a rate ($k_{obs} = 0.10 \text{ min}^{-1}$) equivalent to that of 8-mercaptop-FAO,\(^2\) suggesting that the protein-bound flavin was in fact the reactive species and that position 8 was available to solvent.

\(^1\) The intrinsic fluorescence of 8-SCH$_2$CONH$_2$-FMN appeared upon its release from flavodoxin, in direct analogy to FMN. Upon release from flavodoxin, the nonfluorescent 8-mercaptop-FMN was rapidly converted to the fluorescent 8-SCH$_2$CONH$_2$-FMN by the excess iodoacetamide present in the medium.

\(^2\) R. Hille, J. A. Fee, and V. Massey, manuscript submitted for publication.
than the 8-mercapto group. The fast phase might have conceivably been due to a direct inhibitory effect of iodoacetamide (0.5 mM after dilution into the catalytic assay mixture) on the catalytic reaction and not to a protein modification. It was found, however, that when present initially, 0.5 mM iodoacetamide had no effect on the catalytic assay. These findings raise the possibility that the protein with thiol group(s) derivatized might possess an altered structure around position 8 of the flavin, and that the apparent availability of position 8 to solvent is not necessarily the case with derivatized enzyme. With this caution in mind, we can only tentatively suggest that position 8 of the flavin on native D-lactate dehydrogenase is available to solvent.

Case 4, 8-Mercaptoluciferase—At 4 °C, this enzyme exhibited a slow but measurable reaction with iodoacetamide \( (k = 0.0030 \text{ min}^{-1}) \), with 12.5 mM iodoacetamide and 15.8 \( \mu \text{M} \) excess apoluciferase. The comparable reaction with 8-mercapto-FMN at 4 °C was 30-fold faster \( (k = 0.096 \text{ min}^{-1}) \). Since 8-mercapto-luciferase exhibits the characteristic blue absorbance spectrum of the quinonoid mesomer (data not shown), it seemed reasonable to suggest that the observed reaction represented release of 8-mercapto-FMN and its subsequent reaction with iodoacetamide free in solution. To substantiate this interpretation, the observed rate was calculated as described under "Materials and Methods" (Equation 1). The dissociation rate \( (k_d = 3.4 \text{ min}^{-1}) \) for 8-mercapto-FMN release from 8-mercaptoprotein was determined by following the loss of absorbance due to 8-mercapto-luciferase (at 595 nm) which occurred upon mixing 7.2 \( \mu \text{M} \) 6-OH-FMN with 2.6 \( \mu \text{M} \) 8-mercapto-luciferase. The association rate \( (k_a = 1.7 \times 10^5 \text{ M}^{-1} \text{ min}^{-1}) \) was calculated from the dissociation rate and the dissociation constant \( (K_d = 2 \times 10^{-7} \text{ M}, \text{determined by equilibrium titration}) \). The calculated value for the \( k_{obs} \) \( (k_{obs} = 0.0012 \text{ min}^{-1}) \) was in good agreement with the measured value \( (k_{obs} = 0.0030 \text{ min}^{-1}) \), supporting the contention that iodoacetamide does not react with luciferase-bound 8-mercapto-FMN.

8-Mercaptoriboflavin-Binding Protein—This protein was not tested with the thiol reagents because it has been shown that riboflavin-binding protein preferentially binds the protonated (hence unreactive) form of 8-mercaptoriboflavin (20).

**Consideration of the Enhanced Reactivity of Protein-bound Flavin over Free Flavin**

In the course of these investigations, we have repeatedly encountered instances in which the reaction rate with the flavoprotein was greater than the rate with the free flavin under identical conditions. This was equally true whether the flavin served as a nucleophile (8-mercapto form) or as the substrate for a nucleophile (8-Cl form). Since a multitude of factors interact to define the observed rate for a nucleophilic reaction with a nucleophile (or a substrate for a nucleophile) suggests that protein enhancement of the flavin nucleophility is not the dominant factor, i.e. increased polarizability, basicity, or electronegativity of the flavin itself. Although different factors could be operating in different cases, the variety of enzymes and reactions appears to require some factor which will generally enhance nucleophilicity. A decreased solvation of the reactants in the protein environment, relative to that in free solution would satisfy this requirement (45). Since the isoalloxazine-binding domain of flavoproteins is generally believed to be nonpolar, entering this region would be expected to result in a decrease in solvation. The type of reactions we used (negatively charged nucleophiles and neutral reaction partners) are predicted to give a rate enhancement upon changing to a less polar environment (46). Although changing to a less polar medium should not generate "large" rate enhancements, especially with large nucleophiles such as those we have used, the rate enhancements which we find (1.5- to 10-fold) are "small" on the chemist's scale and consistent with a solvation effect. We believe therefore that the increased rates found with many flavoproteins are due to decreased polarity of the protein environment in the vicinity of flavin position 8.

**Effect of Ligand Binding on the Solvent Accessibility of Position 8**

Binding of ligands to protein is typically accompanied by a change in the protein conformation. The extent to which this conformational change alters the active site is normally difficult to assess. With the method employed above we can approach this question directly. The reactivity of position 8 towards solvent-borne reagents for liganded versus unliganded flavoproteins should be sensitive to structural changes in the protein around flavin position 8. Although interpretation of the results may be difficult if the reactivity differences are small (less than a factor of 10, cf. preceding section), a large decrease in activity would indicate blocking of position 8 while a large increase would suggest increased exposure of position 8 to solvent. We have examined the effect of ligand binding with four flavoproteins which were found to have solvent accessible position 8 in the unliganded state (see Tables I and II). In each case, ligand binding to the protein was monitored by changes in the flavoprotein absorbance spectrum and sufficient ligand was added to ensure saturation.

With 8-Cl-d-aminooxidase, 6.3 mM benzoate caused reactivity with both sulfide (12.5 mM) and thiophenol (0.25 mM) to be reduced to zero. After 18 h, none of the chloride had been displaced from position 8. This indicates that position 8 of d-amino oxidase is totally blocked upon binding benzoate. Whether this blockage is due to the benzoate molecule directly or to a conformational change in the enzyme, is not clear.

NADPH will bind to 8-mercapto old yellow enzyme without causing reduction of the flavin. In the presence of 59 \( \mu \text{M} \) NADPH, 8-mercapto old yellow enzyme reacted with 12.5 mM iodoacetamide at a pseudo-first order rate \( (k_{obs} = 0.022 \text{ min}^{-1}) \) which was 36-fold slower than the rate for the uncomplexed enzyme \( (k_{obs} = 0.80 \text{ min}^{-1}) \). When 114 \( \mu \text{M} \) NADPH was used, the iodoacetamide reaction occurred at 0.006 min\(^{-1}\), suggesting a competition between NADPH and iodoacetamide for free old yellow enzyme. Thus, compared to uncomplexed enzyme, the position 8 in the enzyme-NADPH binary complex is less accessible (possibly inaccessible) to solvent. As before, we cannot differentiate between a direct blockage of the position 8 by the NADPH molecule or a protein conformational change to block the position 8.

The binary complex of melilolate (0.5 mM) and 8-Cl-melilotate hydroxylase reacted with thiophenol (37 \( \mu \text{M} \)) at a rate \( (k_{obs} = 4.2 \text{ min}^{-1}) \) which was 2-fold faster than the uncomplexed 8-Cl-melilotate hydroxylase. This appears to indicate that liganded enzyme offers a greater accessibility of the position 8 to solvent than unliganded enzyme. However, when melilolate (1.25 mM) was bound to 8-mercaptopmelilotate hydroxylase, the reaction with iodoacetamide (12.5 mM) occurred at the same rate \( (k_{obs} = 0.23 \text{ min}^{-1}) \) as was found with the uncomplexed enzyme. The rate increase seen with li-
ganded 8-C1-melilotate hydroxylase must now be interpreted with caution. Ligand-induced changes in the environment of the position 8 might alter the nucleophilic reactivity, thereby causing small changes in the observed rate which are unrelated to changes in the physical accessibility of position 8 to solvent.

A similar situation occurred with p-OH-benzoate hydroxylase. In the presence of 1.5 mM p-OH-benzoate, the second order rate constant for the reaction of iodocetamide with 8-mercaptop-p-OH-benzoate hydroxylase was increased from $k = 114$ m$^{-1}$ min$^{-1}$ to $k = 603$ m$^{-1}$ min$^{-1}$. However, the rate for thiophenol (0.12 mM) reacting with 8-C1-p-OH-benzoate hydroxylase ($k_{obs} = 0.47$ min$^{-1}$) was the same in the presence and absence of 2.5 mM p-OH-benzoate. In this case, it is possible that the presence of the ligand caused a rise in the $pK_a$ of the flavin thiol in 8-mercaptop-p-OH-benzoate hydroxylase (free solution $pK_a = 3.8$ (21)) thereby increasing the nucleophilicity of the thiolate anion and hence the observed rate of reaction. On the other hand, 8-C1-p-OH-benzoate hydroxylase would be expected to be insensitive to this ligand-induced effect since it is not the nucleophile. In this way, the observed rate increase can be rationalized without invoking a change in the accessibility of position 8 to solvent.

**SUMMARY**

In the course of this work, we have accumulated strong evidence indicating that position 8 of the flavin in the following flavoproteins is accessible to solvent: melilotate hydroxylase, p-amino acid oxidase, old yellow enzyme, p-OH-benzoate hydroxylase, and flavodoxin. In addition, we have offered evidence which indicates that position 8 of the flavin of luciferase, p-lactate dehydrogenase, and xanthine oxidase may be accessible to solvent. We have also described negative results which are consistent with the suggestion that position 8 of the flavin of the following flavoproteins is protected from solvent: l-lactate oxidase, glucose oxidase, putrescine oxidase, and riboflavin binding protein. In addition, we have demonstrated that binding benzotri to p-amino acid oxidase and binding NADPH to old yellow enzyme results in blockage of the previously accessible position 8 on both of these protein-bound flavins. Complexing melilotate hydroxylase or p-OH-benzoate hydroxylase with their natural substrates (melilotate and p-OH-benzoate, respectively) causes only minor changes in the rate of reaction. On the other hand, 8-C1-p-OH-benzoate hy-