J. Am. Chem. Soc. **1981**, *103*, 182-184

Oxidation of Dithiols by Flavopapain

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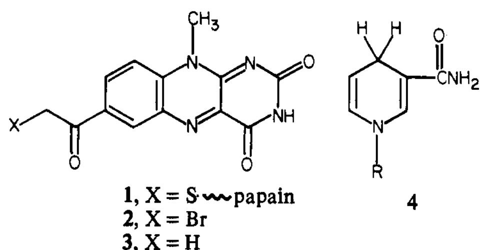
Abstract: The redox reactions between flavopapain **1** and various dithiols have been examined under anaerobic conditions. This semisynthetic enzyme is an effective catalyst relative to the model flavin, 7-acetyl-10-methylisoalloxazine (**3**). Relative rate enhancements, $(k_{cat}/K_m)/k_2^{model}$, observed are 3.9, 8.0, and 17.4 for dithiothreitol, *d,l*-dihydrolipoic acid, and *d,l*-dihydrolipoamide, respectively. This substrate specificity, also seen in the *N*-alkyl-1,4-dihydronicotinamide series, is explicable in terms of favorable hydrophobic-hydrophobic binding interactions between the substrates and the enzyme active site. Stereospecificity was not observed in the enzymatic reactions of the dithiols. It is demonstrated that the enzyme and model flavin reactions of the dithiols both proceed via comparable mechanistic pathways.

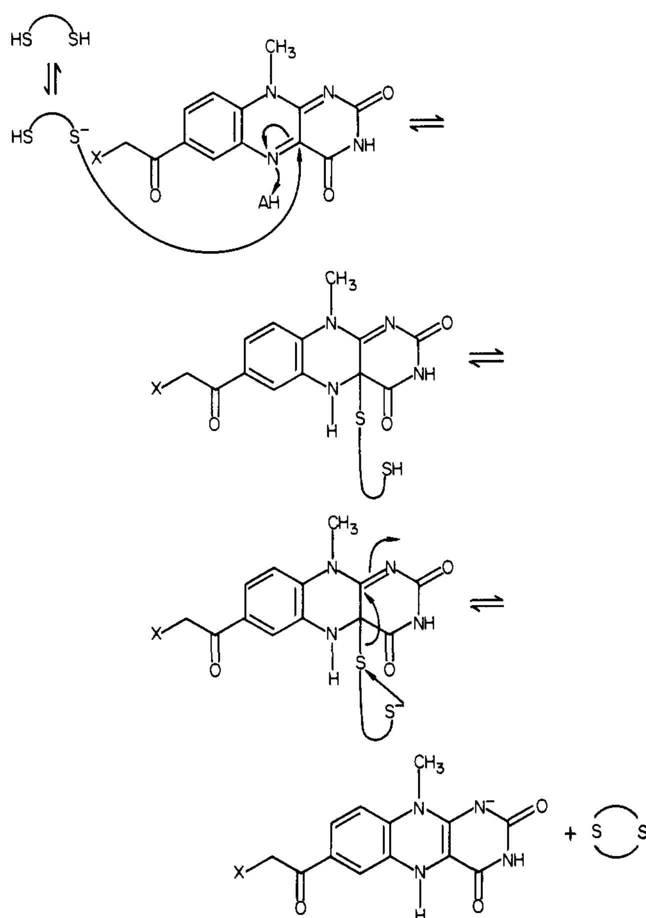
Introduction

We have recently been engaged[1-5] in the design of new catalytic species derived from the modification of enzyme active sites with reactive coenzyme analogues. Toward this goal, we have successfully prepared several covalently bound coenzyme-enzyme-modified complexes of papain and chymotrypsin by using various flavin derivatives. To date, the most efficient semisynthetic enzyme examined is flavopapain **1**, generated from the reaction of 7-(α-bromoacetyl)-10-methylisoalloxazine (**2**) with the essential thiol group of Cys-25 of papain. Previously, we have reported[1,3-5] that flavopapain **1** is an effective oxidoreductase in its reactions with dihydronicotinamide derivatives **4**. This flavoenzyme exhibits saturation kinetics at low substrate concentrations and, relative to 7-acetyl-10-methylisoalloxazine (**3**), modestly accelerates the rate of dihydronicotinamide oxidation.



In view of papain's broad specificity, it appeared likely that flavopapain **1** would be able to bind other classes of substrates and behave as a catalyst in new redox reactions. The oxidation of dithiols by flavins to produce disulfides is an important and interesting biological process which seemed to be well suited for our purposes. The reactions of dithiols with model flavins have been studied extensively under anaerobic conditions. Gascoigne and Radda[6] first examined the reaction of dihydrolipoic acid with various flavin derivatives and found the reaction to be first order in flavin and in dithiol. Further studies[6-9] have established that the reaction of flavin and dithiol (to yield reduced flavin and disulfide) is a general one that exhibits buffer catalysis and is pH dependent. The currently accepted mechanism, Scheme I,[10] involves a rate-determining nucleophilic addition of a thiolate ion to the C(4a) position of the flavin. This covalent adduct formation is assisted by general-acid catalysis at N(5). Ionization of the remaining thiol group followed by nucleophilic attack on sulfur occurs in a rapid step, generating the disulfide and reduced flavin anion.

Scheme I. Postulated Mechanism of Flavin-Catalyzed Oxidation of Dithiols as Applied to the Reactions of Flavins **1-3**



In this article, we examine the oxidation of dithiothreitol (DTT), *d,l*-dihydrolipoic acid, and *d,l*-dihydrolipoamide by flavopapain **1** and flavin **3**.

Experimental Section

Instrumental Data. Spectrophotometric determinations were performed on Beckman Acta MVI and Varian Cary 219 spectrophotometers

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(10) Another mechanistic process involving nucleophilic addition of S S to the flavin has been omitted for simplicity.

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equipped with thermostatic baths holding the temperature in the cell compartments to 25.0 ± 0.3 °C. High-pressure liquid chromatography (high-pressure LC) experiments were carried out on a Waters Associates liquid chromatograph equipped with a Du Pont C-18 Zorbax 8 50952702 column. Electron paramagnetic resonance (EPR) spectroscopy experiments were conducted on a Varian *E*-Line EPR spectrometer. Amino acid analyses were performed by using a Beckman Model 121 automatic amino acid analyzer.

Materials. The following materials were purchased from the companies indicated: *d,l*-lipoamide, *d,l*-lipoic acid, *d,l*-dihydrolipoic acid, Sigma; dithiothreitol (DTT) and *N*-benzoyl-*d,l*-arginine *p*-nitroanilide (BAPA), Aldrich; tris(hydroxymethyl)aminomethane (Tris), Schwarz-Mann. Buffers were prepared with doubly distilled deionized water (Continental Demineralizer). All other solvents were of the highest purity available and were used without further purification. Papain used throughout this work was obtained from Sigma as a suspension of a twice recrystallized enzyme in 0.05 M acetate, pH 5.0.

7-Acetyl-10-methylisoalloxazine (3) and 7-(α-bromoacetyl)-10-methylisoalloxazine (2). These compounds were synthesized from *p*-chloroacetophenone as previously described.[3]

***d,l*-Dihydrolipoamide (*d,l*-6,8-Dimercaptooctanoic Acid).** This compound was prepared from *d,l*-lipoamide according to the procedure of Reed and co-workers.[11] The resulting white crystals (mp 64–66 °C (lit.[11] 66–70 °C)) were repeatedly recrystallized from toluene/petroleum ether and used immediately after preparation.

Preparation of Flavopapain (1). Commercially available papain was purified as its mercury derivative as previously reported.[3] The purified enzyme was then activated and modified with bromoflavin **2** as before.[3] For experiments requiring more concentrated solutions of **1**, solutions of mercuripapain were passed through ultrafiltration membranes (Amicon Corp.), collected, and modified as above. Anaerobic solutions of flavopapain were obtained by dialyzing the enzyme against oxygen-free deionized water. The concentrations of solutions of **1** were determined by using ϵ_{427} = 10 900 M^{-1} cm^{-1}.[3]

Kinetics. All kinetic measurements were carried out at 25.0 ± 0.3 °C at a calculated ionic strength of 0.1 (adjusted with KCl) unless otherwise stated. The reactions were followed spectrophotometrically by monitoring the reduction of the flavin moieties in **1** or **3** at 427 nm under pseudo-first-order conditions with dithiol in excess. In a typical experiment, 3 mL of a buffered solution (0.02 M Tris–HCl, pH 7.5) containing the flavin moiety (10^{-4}–10^{-6} M) was added to the main compartment of a modified Thunberg cuvette (placed in a drybox under nitrogen atmosphere). The dithiol, dissolved in dimethyl sulfoxide (or in water in the case of DTT), was added from the side arm of the cuvette to initiate the reaction after the system had equilibrated to 25.0 °C in the spectrophotometer. Upon completion of these reactions, admittance of O_2 regenerated the oxidized flavin species quantitatively. The pH values of the final solutions were confirmed after each run.

For experiments conducted in the presence of a tenfold excess of flavopapain, reactions were monitored by observing the decrease in absorbance at 427 nm. Finally, for kinetics performed in the presence of oxidized disulfide, *d,l*-lipoamide was incubated with **1** for 10–30 min prior to the addition of *d,l*-dihydrolipoamide.

Product Isolation Experiments. Under kinetic conditions, flavopapain (7 × 10^{-5} M) was treated with 1.1 equiv of *d,l*-dihydrolipoamide. Simultaneously, a control solution was prepared containing all of the reagents except **1**. The redox reaction was allowed to proceed for 6 half-lives, after which each solution was quenched by the addition of 0.3 mL of concentrated H_2SO_4 (final pH ~1). Immediately upon addition of the acid, flavopapain precipitated from solution. The denatured enzyme was removed by filtration, and an aliquot of the resulting filtrate was immediately injected into the high-pressure LC instrument. At a detector wavelength of 230 nm, employing a solvent system of 60% water/40% methanol on a reverse phase C-18 column, both the starting dithiol and oxidized product are easily characterized. High-pressure LC analysis of the solution in the vial containing the enzyme showed, within experimental error, complete conversion of the dithiol to *d,l*-lipoamide. In contrast, analysis of the control solution revealed a major peak corresponding to *d,l*-dihydrolipoamide and a smaller one having the same retention time as the oxidized product.

Experiments to Determine the Stability of Papain's Disulfide Linkages. (a) Trapping with Iodoacetate. Under kinetic conditions, a 100-fold excess of *d,l*-dihydrolipoamide was allowed to react with flavopapain **1** for 20–30 half-lives. Following this period, a large excess of sodium iodoacetate was added to the reaction solution and allowed to stand for 1 h. The enzyme was desalted on a Sephadex G-25 column, lyophilized, hydrolyzed (in a sealed tube containing 6 N HCl for 24 h at 110 °C), and subjected to amino acid analysis. No evidence for the presence of carboxymethylcysteine was obtained.

(b) BABA Hydrolysis Assay[12] of Papain in the Presence of Excess *d,l*-Dihydrolipoamide. In the main compartment of a modified Thunberg cuvette was placed 2.0 mL of buffer containing 0.1 M Tris–HCl (pH 7.5) and 1 mM EDTA, 0.1 mL of 0.174 M *d,l*-dihydrolipoamide (in dimethyl sulfoxide), and 0.1 mL of a purified papain solution (1–3 × 10^{-5} M in water). After the dithiol had incubated with the enzyme for 45 min, 0.025 mL of 0.1 M BAPA in dimethyl sulfoxide was added from the side arm of the cuvette. The rate of *p*-nitroaniline release, monitored at 410 nm, was found to agree within experimental error with the results from a control experiment conducted in the absence of the dithiol.

Table I. Second-Order Rate Constants for the Oxidation of Dithiols by Flavopapain **1** and by 7-Acetyl-10-methylisoalloxazine (**3**)

dithiol	model reactn k_2^{model}, M^{-1} s^{-1}	enzymatic reactn k_{cat}/K_m, M^{-1} s^{-1}	enzymatic rate enhancement
dithiothreitol[a] (DTT)	0.99	3.86	3.9
d,l-dihydrolipoic acid[b]	0.84	6.70	8.0
d,l-dihydrolipoamide[c]	1.21	21.0	17.4

[a] Measured at 25 °C in 0.02 M Tris–HCl containing 0.1 mM EDTA (pH 7.5, μ = 0.1 (adjusted with KCl)). [b] Same conditions as for *a* except pH 7.3. [c] Same conditions as for *a* except with 3–6% dimethyl sulfoxide (v/v).

Results and Discussion

Under substrate in excess conditions the reaction of DTT, *d,l*-dihydrolipoic acid and *d,l*-dihydrolipoamide with 7-acetyl-10-methylisoalloxazine (**3**) yielded linear pseudo-first-order plots well beyond 5 half-lives. As previously reported[6-9] for the reactions of flavin derivatives with dithiols, the above reactions were also found to be first order in both species. Thus, plots of pseudo-first-order rate constant (k_{obsd}) against dithiol concentration exhibited linearity. The second-order rate constants appearing in Table I were obtained by dividing k_{obsd} by the total dithiol concentration.

In the dithiol oxidations by flavopapain **1**, pseudo-first-order plots were linear for 70–95% of the reaction followed by upward deviations for the remainder of the reaction. In order to obtain accurate k_{obsd} constants, we determined A_∞ values graphically by using the method of Kězdy.[13] Pseudo-first-order plots obtained in this way displayed excellent linearity. Plots of these k_{obsd} values against the total dithiol concentration were linear, indicating that these reactions also were first order in flavoenzyme and in dithiol. Since the redox reactions were conducted under nonturnover conditions (the flavin moiety remains reduced in the absence of O_2), the flavoenzyme is not regenerated in its catalytically active oxidized form. Hence, the second-order rate constants appearing in Table I for the enzymatic reactions were determined as described for the model oxidation reactions. In all cases, the values reported are larger than those for the corresponding model reactions and were invariant throughout many flavopapain preparations.

Several experiments were conducted to determine whether the deviation from pseudo-first-order behavior in the enzymatic reactions was mechanistically significant. It was of primary concern to exclude the possibility of intermediate formation during the course of the flavin reduction. This situation would correspond kinetically to a rapid formation of an intermediate (resulting in bleaching of >75% of the flavin chromophore) followed by a much slower breakdown of the intermediate to yield the reduced flavin.

One plausible intermediate would be a stable covalent adduct arising from thiolate ion addition to the C(4a)-position of the flavin. Although in model systems such adducts are known to break down rapidly[6-9] to form the disulfide and reduced flavin, steric constraints within the active site of the enzyme may render

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this process more difficult. An experiment that eliminates this possibility is the examination of the kinetics of flavoenzyme reduction under conditions of enzyme in excess. In this situation pseudo-first-order plots were found not to deviate from linearity. The calculated second-order rate constants, determined by dividing k_{obsd} by flavopapain concentration, were found to agree within experimental error with the enzyme k_{cat}/K_m values reported in Table I. Furthermore, the formation of a stable covalent adduct is unlikely since admittance of O_2 after several half-lives of reactions with dithiol in excess quantitatively regenerates the spectrum of the oxidized flavin species. Finally, product isolation studies were conducted for the reaction of **1** and *d,l*-dihydrolipoamide. Flavopapain was allowed to react with 1.1 equiv of the dithiol for roughly 6 half-lives. Anaerobic workup followed by high-pressure LC analysis revealed that 90 ± 10% of the expected product, *d,l*-lipoamide, was formed.

It would be desirable to examine the dithiol oxidation reactions under turnover (aerobic) conditions and monitor the rate of disulfide formation. If the rate of disulfide formation corresponded to the rate of flavopapain reduction determined anaerobically, strong evidence that the enzymatic redox reactions do not involve stable intermediates would be provided. Unfortunately, the presence of O_2 and H_2O_2 (formed from the reaction of reduced flavin and O_2) interfere with the kinetics of these reactions.[14] Despite these difficulties, initial rate experiments were conducted aerobically by following the appearance of *d,l*-lipoamide at 325 nm. Qualitatively it was evident that the enzymic oxidation process was at least 1 order of magnitude faster than that of the dithiol–**3** reaction.

Another mechanistic pathway that must be considered in the enzymatic reactions is the formation of a long-lived flavin radical since flavin semiquinones are known to exhibit high stability.[15] To address this point, ESR spectra were recorded during the redox reaction of **1** and *d,l*-dihydrolipoamide under conditions where flavin radicals have been characterized.[16] A concentrated solution of flavopapain ($\sim 5 \times 10^{-5}$ M) was treated with 1 equiv of the dithiol in a flat cell under a nitrogen atmosphere. An ESR spectrum was taken immediately, and measurement of ESR spectra continued every 10 min for a total period of 1.5 h ($t_{1/2}$ of reaction $\sim$14 min under these conditions). Although 1% of the flavin semiquinone should have been detectable, no ESR signal was observed.

Product Inhibition Experiment. A process that could account for the observed enzyme kinetics would involve strong product inhibition. In this situation, the disulfide product would have to bind significantly tighter to the enzyme's active site than the starting dithiol. This possibility can be ruled out, however, since preincubation of **1** with a large excess of *d,l*-lipoamide had no significant effect on the kinetics of the *d,l*-dihydrolipoamide oxidation.

Papain Denaturation Experiments. Another potential side reaction in the flavopapain–dithiol redox reactions could involve denaturation of the enzyme. This event could arise from reduction of one or more of papain's three disulfide linkages by the strongly reducing dithiols employed. In one experiment, *d,l*-dihydrolipoamide was incubated with **1** for more than 20 half-lives. The final solution was then treated with excess iodoacetate and the enzyme isolated and finally subjected to amino acid analysis. Carboxymethylcysteine, the expected product if disulfide reduction had occurred, was not detected.

The stability of the disulfide linkages of native papain toward reduction by DTT or dihydrolipoamide was also examined. Purified papain was incubated with excess dithiol for nearly 1 h under anaerobic conditions. At that time, BAPA was added and the rate of *p*-nitroaniline release monitored. Papain's hydrolytic activity measured in this way was found to agree well with that of assays performed in the absence of dithiol.

The above experiments suggest that deviation from linearity in the enzymatic pseudo-first-order plots is probably due to the existence of secondary reactions. It is conceivable that small quantities of poorly active flavopapain are present in our enzyme preparations. This situation could arise from the presence of small amounts (<1%) of unmodified active papain that is still capable of autoproteolytic behavior. Another possibility is that during the enzyme modification procedure, bromoflavin **2** may have, to a very minor extent, reacted with other amino acid residues (besides Cys-25) in the protein. In either case, under the nonturnover conditions employed, the simultaneous reduction of poorly reactive flavin moieties in addition to active **1** could produce the multiphasic kinetics observed. The kinetics performed under conditions of enzyme in excess support this hypothesis since active flavopapain will competitively react with the limiting dithiol reagent and yield monophasic kinetics.

Substrate Selectivity. Comparison of the second-order rate constants for the model flavin and enzyme reactions reveals that the latter are significantly larger (Table I). For DTT, flavopapain **1** is 3.9 times more efficient as an oxidizing agent than 7-acetyl-10-methylisoalloxazine **3**. The more apolar substrate, *d,l*-dihydrolipoic acid, appears to bind tighter to this flavoenzyme's hydrophobic active site. This effect is reflected by an eightfold rate enhancement relative to **3** in the enzymatic reaction. When the charged carboxyl group of this substrate is replaced by the neutral amido moiety, stabilizing hydrophobic–hydrophobic interactions[3,5] between **1** and *d,l*-dihydrolipoamide appear to increase, affording a 17.4-fold enzymic rate enhancement.

The trend of increasing the rate of the enzymatic oxidation reactions by increasing the hydrophobicity of the substrates has also been observed in flavopapain's reactions with *N*-alkyl-1,4-dihydronicotinamides (**4**).[5] For *N*-ethyl-, *N*-propyl-, and *N*-hexyl-1,4-dihydronicotinamide, the k_{cat}/K_m values determined from the aerobic reactions with **1** were 5590, 8100, and 10480 $M^{-1}\ s^{-1}$, respectively. In addition, K_m values for these substrates decrease with increasing alkyl chain length (1.3, 1.0, and 0.42×10^{-5} M, respectively), suggesting that binding efficiency is enhanced with the more hydrophobic substrates.

Substrate Stereospecificity. In the aerobic oxidation of stereospecifically labeled NADH (with deuterium in the 4A- and 4B-positions) flavopapain **1** was found to display a sevenfold preference for catalyzing the transfer of hydrogen from the 4A side of the dihydronicotinamide.[4] In view of this result, one might expect that one of the enantiomers of *d,l*-dihydrolipoamide would be preferentially oxidized by **1**. The kinetic results from experiments with enzyme in excess indicate that this is not the case, since a monophasic reaction accompanied by the expected amount of flavoenzyme reduction was observed. The reason for the lack of stereospecificity in this reaction is probably related to the poor binding of dihydrolipoamide to **1**. Inefficient substrate binding is evident since saturation kinetics were not detected at dithiol concentrations as high as 5 mM. In addition, estimates from initial rate experiments place the K_m value of this dithiol with **1** at roughly 5×10^{-2} M. In contrast, NADH binds to **1** nearly 2 orders of magnitude more efficiently, as suggested by its K_m of 2.2×10^{-4} M. Lastly, preliminary experiments have shown that flavopapain exhibits no significant preference for dithioerythritol (DTE) relative to its diastereomer, DTT.

Acknowledgment. Our work was supported in part by National Science Foundation Grants AER 77-14529 and DAR 7910245 (E.T.K) and by NIH Postdoctoral Fellowship GM 07542 (H.E.F.) We wish to thank Mr. Eugene V. Sitzmann for carrying out the EPR experiments.

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