FLAVOPAPAIN: SYNTHESIS AND PROPERTIES OF SEMI-SYNTHETIC ENZYMES

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Summary: The alkylation of the sulfhydryl group of Cys-25 in the active site of papain by 8α-bromo-2',3',4',5'-tetra-O-acetylriboflavin, 2, and 7α-bromoacetyl-10-methyl isoalloxazine, 5, produces two new semi-synthetic oxidoreductases 3 and 6, respectively, which are capable of oxidizing N-benzyl-1,4-dihydronicotinamide, 7. While the reactivity of flavopapain 3, as measured by k_{cat}/K_m, is comparable to that of a corresponding model flavin, 1, the k_{cat}/K_m value of 5900 $M^{-1}sec^{-1}$ at 25.0° measured in the case of flavopapain 6 is considerably larger than the second order rate constant of 121 $M^{-1}sec^{-1}$ for the related reaction of the model flavin 7-acetyl-10-methylisoalloxazine, 4.

The large scale use of oxidoreductase enzymes as stereospecific catalysts in synthetic organic chemistry is hampered by the fact that these enzymes are often neith readily available nor adequately characterized* (1). One means of avoiding these difficulties would be to attach a redox coenzyme analog covalently at or near the active site of a hydrolytic enzyme and thereby synthesize a new easily accessible semi-synthetic oxidoreductase. If the substrate binding properties of the original hydrolytic enzyme are not greatly altered upon modification by such an analog, then the new enzy should behave like any other natural enzyme, exhibiting substrate binding, rate accele tions, and stereospecificity in its reactions. The cysteine protease, papain (E. C. 3.4.4.10), is known to possess an active site groove some 25 Å in length (2). Furthe more, several reports in the literature (3) suggest that blockage of the active site sul hydryl group present at Cys-25 does not prevent binding of peptides at the active site of the enzyme. Papain is thus ideally suited for the modification described here.

*Horse liver alcohol dehydrogenase is a notable exception (1).

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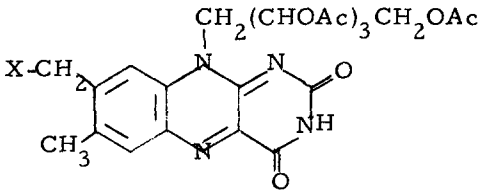
In the present communication the preparation of two semi-synthetic oxidoreductases, flavopapain 3 and flavopapain 6, by reaction of the sulfhydryl group of Cys-25 in the active site of papain with the brominated flavins 2 and 5 is reported. Also, the results of a kinetic study of the reaction of modified papains 3 and 6 and the model flavins 2',3',4',5'-tetra-O-acetylriboflavin, 1, and 7-acetyl-10-methylisoalloxazine, 4, with N-benzyl-1,4-dihydronicotinamide, 7, are described.



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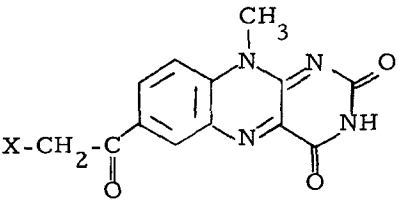
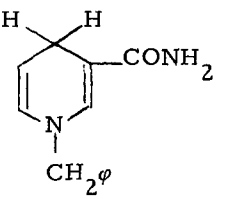
2 X = Br
3 X = S-papain



4 X = H
5 X = Br
6 X = S-papain



7

RESULTS AND DISCUSSION

Synthesis of Flavin Derivatives

2',3',4',5'-Tetra-O-acetylriboflavin, 1, and 8α-bromo-2',3',4',5'-tetra-O-acetylriboflavin, 2, were prepared from riboflavin (Aldrich) by the method of Falk et al. (4). 7-Acetyl-10-methylisoalloxazine, 4, and 7α-bromoacetyl-10-methylisoalloxazine, 5, were prepared by a procedure to be published elsewhere (5).

Modification of Papain

Twice recrystallized papain (Worthington Biochemical Co.) was purified by affinity chromatography (6). For modification, the freshly purified papain in deionized water was mixed with a 5-fold molar excess of brominated flavin in acetone or DMSO at room temperature. After 5 hr of incubation, a 5-fold molar excess of cysteine was

added, followed by a second addition of brominated flavin. Another addition of cysteine and brominated flavin was made 5 hr later (a total of three additions), and the solution was left to stand at room temperature for at least another 5 hr. The modified papain, which exhibited less than 1% activity toward either N-benzyloxycarbonylglycine p-nitrophenyl ester (7) or N-benzoyl-D, L-arginine p-nitroanilide (8) was then separated from excess brominated flavin by dialysis against deionized water (8 changes in 3 days) at 4°C. The multiple additions described above for the modification of papain were necessary due to the fact that a small amount of Cys-25 thiol-C-4a flavin adduct, similar to that reported by Loechler and Hollocher (9) in the oxidation of dithiothreitol, is formed when only one addition of brominated flavin is made. The addition of cysteine catalyses the decomposition of this adduct releasing active unmodified papain. The site of modification of the enzyme was determined by S-carboxymethylation followed by amino acid analysis (10). In both flavopapains, no S-carboxymethylcysteine was detected indicating that papain has been modified exclusively at Cys-25.

Oxidation of 7 by Flavins 1 and 4

The oxidation of dihydronicotinamides by flavin has been reported to proceed via pre-equilibrium charge transfer complexes (11). The dissociation constants for the complexes formed by various flavins are on the order of 0.1 M (11c). Therefore, if one maintains conditions in which the concentrations of both reagents are much less than 0.1 M, complex formation is negligible, and the reaction can be treated as a simple second order reaction. In our experiments, the concentrations of the flavins and the dihydronicotinamide were never greater than 0.001 M, and no evidence for complex formation was observed.

The results of measurements on the rate of oxidation of 7 by flavins 1 and 4 both aerobically and anaerobically are shown in Table I. For each flavin, the aerobic and anaerobic rate constants agree within experimental error. The greater rate of

TABLE I

Oxidation of 7 by Flavin Derivatives[a]

Flavin	Aerobic[b] (M^{-1} sec^{-1})	Anaerobic[c,d] (M^{-1} sec^{-1})
1	57.2 ± 4.9	52.3 ± 6.8
4	121 ± 28	97.0 ± 16.5

[a] Measured in 0.1 M Tris-HCl buffer (0.5% ethanol, v/v), pH 7.5, 25.0 ± 0.03°C.

[b] Calculated from $k_{obs} = k[\text{Flavin}]$ where k_{obs} is the pseudo-first order rate constant obtained from a plot of log $(A_t^{355} - A_\infty^{355})$ vs t.

[c] Calculated from $k_{obs} = k[7]$ where k_{obs} is the pseudo first order rate constant obtained from a plot of log $(A_t^{447} - A_\infty^{447})$ vs t (for 1) or log $(A_t^{427} - A_\infty^{427})$ vs t (for 4).

[d] Anaerobic conditions were maintained by adding glucose oxidase, catalase and glucose to the reaction solutions. (See S. Nakamura and T. Kimura, J. Biol. Chem., 247, 6462 (1972)).

oxidation of 7 by 4 as compared to 1 reflects the former flavin's higher redox potentia (-106 mV (12) vs -179 mV (4)).

Oxidation of 7 by Flavopapains 3 and 6

The aerobic oxidation of 7 by the flavopapains should obey the scheme of equat 1,

$$E_{ox} + BNAH \underset{K_m}{\rightleftharpoons} ES \xrightarrow{k_{cat}} EH_2{}_{red} + BNA$$
$$EH_2{}_{red} \xrightarrow{k_o(O_2)} E_{ox} \qquad (1)$$

where E_{ox} and $EH_2{}_{red}$ represent oxidized and reduced flavopapain, BNAH and BNA

represent 7 and its oxidized form N-benzylnicotinamide, and ES represents a Michaelis complex between substrate and enzyme. Assuming that under the aerobic conditions used, the oxidation of 7 is independent of oxygen, i.e., $k_o(O_2) >> k_{cat}$, then the scheme of equation 1 leads to the expression of equation 2 for the rate of oxidation of 7.

$$v = \frac{k_{cat}[E_{ox}]_o[BNAH]}{K_m + [BNAH]} \qquad (2)$$

Plots of 1/v vs 1/[BNAH] allow the values of K_m and k_{cat} to be determined. At pH 7.5 in 0.1 M Tris-HCl buffer (0-5% ethanol, v/v) and 25.0 ± 0.03° C, the value of k_{cat} for 3 is (5.4 ± 0.9) x 10^{-3} sec^{-1} and that of K_m is (9.7 ± 3.3) x 10^{-5} M. In the case of 6, the corresponding values are (0.13 ± 0.03) sec^{-1} and (2.2 ± 0.8) x 10^{-5} M. Thus, the apparent k_{cat}/K_m values at pH 7.5 for the two flavopapains are 56 $M^{-1}sec^{-1}$ for 3 and 5900 $M^{-1}sec^{-1}$ for 6. Comparing these values to the second order rate constants for the reaction of the free flavins 1 and 4 with 7, one can see that while flavopapain 3 is not a particularly effective catalyst, flavopapain 6 shows rate accelerations of about 50-fold. Furthermore, the k_{cat}/K_m value for flavopapain 6 is comparable to those for the natural flavoenzymes glucose oxidase (13), D-amino acid oxidase (14), and succinate dehydrogenase (15). A significant difference which exists between both flavopapains 3 and 6 and the model flavins 1 and 4 is that saturation kinetics were seen in the reactions of the enzymes with 7 at low concentrations of 7 but not in those of the models.

From the examination of a three dimensional model of papain in which the flavin groups have been covalently attached to the sulfhydryl of Cys-25 the origin of the substantial differences in the reactivity of flavopapains 3 and 6 can be rationalized. In particular, the structures of three papain derivatives produced by reaction of three chloromethyl ketone substrate analogs with the sulfhydryl of Cys-25 have recently been determined by X-ray diffraction (16). In each case the carbonyl oxygen of what was originally the chloromethyl ketone group was found to be near two potential hydrogen

bond donating groups, the backbone NH of Cys-25 and the amide NH_2 of Gln-19. In our own model building,* we oriented the flavin group in flavopapain 6 in a fashion such that the carbonyl group of what was the halocarbonyl function in the modifying agent 5 was brought to a similar position. When this was done it was easily seen tha N-benzyl-1,4-dihydronicotinamide, substrate 7, could be fitted snuggly into a binding pocket in the enzyme in close proximity to the flavin ring, presumably facilitating hydrogen transfer. On the other hand, in the case of flavopapain 3, the carbonyl gro attached to the flavin ring in 6 is not present, and it seems probable that the flavin group is not drawn into the active site region as has been postulated for 6. Of course an additional point which must be considered is that the tetraacetylribose group at the 10-position of the flavin ring in flavopapain 3 is much larger than the methyl group at the 10 position in flavopapain 6, and this steric difference should also contribute to th greater likelihood that the flavin group in 6 will be drawn into the active site region than is the case in 3.

We have not attempted yet to find optimal substrates for the flavopapains. Pr sumably, larger substrates which could interact at more points along the enzyme's large binding crevice will exhibit larger rate accelerations than those seen here. Th substrate specificity and also the stereochemistry of the hydrogen transfer reactions of the flavopapains are under investigation. Finally, the possibility that additional intermediates besides those shown in equation 1 may be present along the reaction pat way in the reduction of flavopapain by 7 is being examined utilizing anaerobic conditio

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*Push-fit molecular models, Labquip, 18, Rosehill Park Est., Reading, U.K.

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