Synthesis and Characterization of a New Semisynthetic Enzyme, Flavolysozyme

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Abstract: The protein template lysozyme has been alkylated with 6α-, 7α-, and 8α-(bromoacetyl)-10-methylisoalloxazine to produce a new class of semisynthetic enzymes, 6-, 7-, and 8-flavolysozyme. Each alkylation reaction produces two flavolysozyme isozymes (I, II) that are distinct in their chromatographic behavior and catalytic reactivity. The properties of isozyme I are consistent with an ester bond between the flavin analogues and Asp-101, a residue on the surface of lysozyme, at the edge of the active site; isozyme II appears to be modified directly in the active site via an ester bond to Asp-52. The substrate specificities of these catalysts contrast with those of all other semisynthetic enzymes. 8-Flavolysozyme II exhibits saturation kinetics during the catalytic oxidation of NADPH and a k_{cat}/K_m value 10-fold higher than the second-order rate constant for the control reaction. The catalytic rate enhancements of 8-flavolysozyme II are most likely generated by substrate–protein interactions and not protein–cofactor interactions since the lysozyme environment does not detectably alter the chemistry of the bound flavin analogue relative to the equivalent free flavin derivatives.

Site-specific mutagenesis is a frequently applied procedure in the protein engineering field that allows the specific replacement of the original amino acid residues in a protein by new residues. The objectives of carrying out such replacements can be to improve the stability of the protein, to change specificity toward substrates, and to elucidate the functional roles of particular amino acids in the active site and structural regions. An alternative and complementary approach pursued in our laboratory involves the introduction of new catalytic functionalities through the selective chemical modification of residues at or near the active sites of enzymes. We have produced semisynthetic enzymes by modifying an amino acid residue of a protein template with a reactive coenzyme analogue. The hybrid species, if properly designed, will exhibit a combination of the general binding properties of the original protein template and the catalytic activity of the covalently bound coenzyme. The ultimate goal of this endeavor is to produce new protein-based catalysts on demand; the criteria necessary for creating these will grow out of the current developmental phase.[1-4] General design guidelines have been set by the production of flavopapain,[1] a redox-active derivative of papain modified at Cys-25 with an α-(bromoacetyl)flavin. The substrate specificity of this synthetic oxidoreductase clearly arises from the hydrophobic crevice proximal to the site of flavin attachment. However, the catalytic efficiency of flavopapain depends on the geometry of substrate–template–cofactor complexes. When these factors are optimized as in the case of a flavopapain described by Radziejewski et al.,[2] the characteristic k_{cat}/K_m values can exceed 10^6 M^{-1} s^{-1}.

To generalize the approach for creating new semisynthetic enzymes, our laboratory has begun to assess other types of proteins that may serve as useful catalytic templates. Clearly, the marked hydrophobicity of papain's active site facilitated the catalytic turnover of hydrophobic substrates dissolved in an aqueous buffer. A formidable challenge was then to develop semisynthetic enzymes that would process hydrophilic substrates in an aqueous environment and thereby expand the potential application of these new catalysts. Results obtained with semisynthetic enzymes based on the attachment of flavin to glyceraldehyde 3-phosphate dehydrogenase demonstrate that in fact a hydrophilic substrate, NADH, can be efficiently oxidized.[3]

This report describes the properties of a new redox-active semisynthetic enzyme based on lysozyme (flavolysozyme) and examines criteria for selecting additional proteins that may serve in the development of novel catalytic species. Lysozyme, a small protein containing a relatively hydrophilic active site, fulfills all the basic requirements for use in the development of a semisynthetic catalyst. It is readily available and quite stable, and its crystal structure[5] and solution properties[6] are well studied. In addition, lysozyme's small size (MW 14000) will allow for future NMR analysis not readily feasible with the large natural flavin-dependent enzymes. Unlike the previous protein templates that have a singularly reactive nucleophile, lysozyme contains only weakly nucleophilic groups. Therefore, a single homogeneous flavolysozyme was not expected to result from a synthetic protocol similar to that used for the production of flavopapain. A procedure for modifying lysozyme was developed to yield isomeric flavolysozymes. These isozymes have been separated, and their catalytic activity has been determined.

Material and Methods

General Procedures. UV–vis and circular dichroic spectroscopies were performed on a Perkin-Elmer λ-5 spectrophotometer and an AVIV 60DS CD spectrometer, respectively.

Materials. Except as noted, all materials were of the highest quality commercially available and used without further purification.

Dihydronicotinamides. Synthesis of the *N*-alkyldihydronicotinamides has been described previously.[7] NMNH,[8] NADH, and NADPH were purchased from Sigma. [4,4-$^2H_2$]NADH was a gift from Dr. Donald Hilvert, this laboratory. *N*-Ribodihydronicotinamide was synthesized by dephosphorylating NMNH (ca. 10 μmols) with *Escherichia coli* alkaline phosphatase (660 units) (Sigma Chemical Co.), pH 8, 37 °C for 1.5 h. This dihydronicotinamide was isolated after the reaction mixture was passed through an anion-exchange column (BioRad AG-2X8).

Lysozyme Alkylation and Flavolysozyme Purification. 1b, 2b, and 3b were synthesized as described earlier[7,9] and used to alkylate hen egg white lysozyme (Sigma Chemical Co.). The alkylating agents (3–4 equiv) were dissolved in a minimum of dimethylformamide and added in four aliquots over 4 h to a solution of 50 mg of lysozyme and 100 mM 2-(*N*-morpholino)ethanesulfonate, pH 5. The bromoacetyl compounds were added in aliquots to afford a maximum yield of flavolysozyme and to overcome the competing hydrolysis reaction of the bromoacetyl group. The reaction mixture was incubated at 60 °C in the dark for a total of 5 h, cooled, and neutralized. Excess flavin was removed by dialysis, gel filtration, or ion-exchange chromatography. The two alkylated products,

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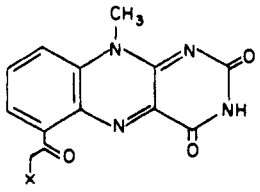
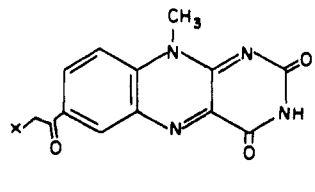
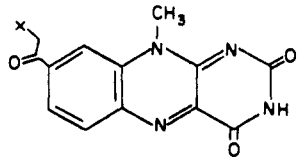
Chart I



1



2



3

a, X = H
b, X = Br

semisynthetic isozymes I and II, and lysozyme were separated on a BioRex 70 cation-exchange column with a 500-mL gradient, 0–500 mM KCl in 50 mM potassium phosphate, pH 7.2. The two flavoproteins isolated from this column were stored at 4 °C for later chemical and physical characterization. The oxidoreductase activity of flavolysozyme II was measured in the presence of the underivatized redox-inert protein template, lysozyme, because the BioRex elution profile for lysozyme and isozyme II overlapped. Flavoprotein concentration was estimated by the extinction coefficient of the appropriate free flavin (3a, ϵ_{440} = 11 040 M^{-1} cm^{-1}). For analytical characterization, isozyme II was separated from isozyme I and lysozyme on a (carboxymethyl)chitin affinity column (CM-chitin) with an acetic acid gradient[10] and on a medium-pressure Mono S (Pharmacia) cation-exchange column with a 0–200 mM KCl gradient in 50 mM potassium phosphate, pH 7.2.

Enzyme Assays. Residual lysozyme activity in a bacterial cell lysis assay was measured by using a suspension of *Micrococcus lysodeicticus* (Sigma Chemical Co.).[11] The oxidoreductase activity of the semisynthetic enzymes and free flavins was studied at 25 °C, pH 8, 100 mM Tris in the presence of catalase (Sigma Chemical Co.) (0.086 mg/mL) and superoxide dismutase (Sigma Chemical Co.) (0.01 mg/mL). The consumption of the dihydronicotinamides was monitored by following their absorbances in the region of 340–360 nm.[7] If 3-(4',5'-dimethylthiazol-2-yl)-3,5-diphenyltetrazolium bromide (MTT) was used as the terminal electron acceptor instead of O_2, the oxidereductase activity was followed at 560 nm (the difference in the ϵ_{560} for the reduced vs. oxidized form of MTT is 14 560).[12] Since flavin derivatives have a basal oxidoreductase activity even when free in solution, the rate enhancements produced by flavolysozyme were always compared to those of the appropriate model flavin system. For this paper, the model rates were measured with the free debrominated flavin derivative isolated after flavolysozyme synthesis.

Reduction and Modification of Flavolysozymes I and II. In an attempt to sequence the flavin-containing peptide fragment of flavolysozyme, lysozyme and isoalloxazine-derivatized lysozyme were denatured, reduced, carboxymethylated, and partially hydrolyzed with trypsin or *Staphylococcus aureus* protease V-8 under standard conditions.[13] The resulting denatured protein and peptides were separated on an analytic C-18 column (Altex) using an aqueous acetonitrile gradient (4–60% in 0.1% trifluoroacetic acid). The K_D values of the sulfite–flavin adducts for the control isoalloxazine and 8-flavolysozymes I and II were determined by the decrease in the cofactor's absorbance (440 nm) at 25 °C, pH 8, 100 mM Tris by using a fresh solution of sodium sulfite.[14] To test for formation of the semiquinone form of the flavin derivative, either free or attached to lysozyme, the general procedure of Massey and Hemmerich was used.[15] The photoreduction was studied in the presence and absence of catalytic amounts of 5-deazariboflavin (the gift of Dr. Christopher Walsh, Massachusetts Institute of Technology).

Results and Discussion

Synthesis and Purification of Flavolysozyme. Flavolysozyme is typically generated by incubating lysozyme and the α-bromoacetyl compound, **1b**, **2b**, or **3b** (Chart I), at 60 °C, pH 5. These conditions are more vigorous than those necessary to convert papain to flavopapain because lysozyme lacks a group that is as reactive as the active-site thiol of papain. To minimize degradation of the isoalloxazine ring system during the synthesis of flavolysozyme, the reaction is typically stopped after 4–5 h at which point ca. 50% of the lysozyme is modified by either 1b, 2b, or 3b.

Since lysozyme does not contain a uniquely reactive nucleophile, the production of flavolysozyme isozymes was predicted and later confirmed when the product mixture was analyzed and separated on a BioRex 70 cation-exchange column. Parsons et al.[16] demonstrated that this resin could separate lysozyme species that were uniquely esterified by triethyloxonium fluoroborate at differing carboxylate groups. The flavin derivatives unattached to protein elute without binding to this cation-exchange column; the two resulting flavolysozymes (I and II) and unmodified lysozyme elute only after a salt gradient is applied to the column. The two isolated flavoproteins are distinct from one another and reproducibly elute in the presence of either low salt (flavolysozyme I) or high salt (flavolysozyme II). After this single chromatographic step, these proteins appear to be homogeneous as judged by their elution profile from a second cation-exchange column (Mono S) and from an affinity column (see below). Flavolysozyme isozymes I and II form in equal amounts at pH 5, and each appears to be modified by only one flavin per polypeptide. When the example of 8-flavolysozymes I and II is used,[17] the A_{280}/A_{440} ratios (ca. 6) are similar to a theoretical ratio of 5.1 calculated from the absorptivity of lysozyme and **3a** (ϵ_{280} of **3a** is 18 800 M^{-1} cm^{-1} and ϵ_{280} of lysozyme is 37 600 M^{-1} cm^{-1} [6]). Therefore, the alkylation of lysozyme produces two unique flavoproteins that are readily separable. No derivative of lysozyme alkylated with more than one isoalloxazine has been isolated.

Characterization of Flavolysozyme Isozymes. The carboxylate groups of the active-site Asp-52 and the surface Asp-101 are known to be the most active nucleophiles of lysozyme at neutral and acidic pH.[18,19] However, the position and type of cofactor–lysozyme bond must be confirmed because of the severity of the alkylation conditions necessary to produce flavolysozyme. As expected, the flavin–lysozyme bond is covalent. After flavolysozyme is denatured, reduced, and carboxymethylated, isoalloxazine and protein still coelute on reversed-phase (C-18) chromatography. This bond was not stable, however, when the denatured protein was subjected to limited proteolysis with trypsin or *S. aureus* V-8 (pH 8). Therefore, peptide sequencing could not be used to identify the exact residues that are alkylated to produce isozymes I or II.

The location and type of the flavin–lysozyme bond were then identified by other chemical and chromatographic techniques. The behavior of flavoprotein (isozyme II) that elutes on a BioRex column adjacent to lysozyme is consistent with expectations for a lysozyme derivative modified in the active site. Flavolysozyme II will not bind to a lysozyme affinity column, CM-chitin, known to interact specifically with lysozyme's active site.[10] The other isozyme, flavolysozyme I, is likely modified at a position outside the active site since it elutes from this affinity column only after a gradient of acetic acid is applied. Furthermore, this species only marginally precedes lysozyme during the gradient elution.

The relative bacterial cell lysis activity of the protein fractions separated by the CM-chitin column reveals two important characteristics of the system. First, flavolysozyme II retains

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Table I. Rate Constants for the Catalytic Oxidation of Dihydronicotinamides by Flavin Derivatives Attached to Lysozyme or Free in Solution[a]

	k_2, M^{-1} s^{-1}				
	free cofactor[b]	8-flavolysozyme I	k_2-(isozyme I)/k_2(cofactor)	8-flavolysozyme II k_2, M^{-1} s^{-1}	k_2-(isozyme II)/k_2(cofactor)
ribodihydronicotinamide	8.6	10	1.2	12	1.4
NMNH	2.9	6.5	2.2	7.7	2.5
NADH	1.4	3.9	2.8	6.1	4.4
NADPH	1.5	8.5	5.7	15[c]	10

[a] These rate constants are an average of the values measured from a range of dihydronicotinamide concentrations (ca. 0.10–1.5 mM) using 1.5 μM catalyst, 100 mM tris, pH 8 (see Materials and Methods). [b] This is the isoalloxazine derivative recovered from the modification of lysozyme. [c] This value, often compared to a second-order rate constant, is actually a k_{cat}/K_m value in which $k_{cat} = 16.1 \times 10^{-3}$ s^{-1} and $K_m = 1.1$ mM.

essentially none (less than 0.5%) of the lytic activity associated with lysozyme, a property consistent with an active-site modification and perhaps specifically a derivatization of Asp-52. Flavolysozyme I maintains 10% of lysozyme's native activity, however. This amount is similar to that found for other lysozyme derivatives modified at Asp-101.[18] Second, lysozyme's native structure is not denatured during the harsh alkylation conditions since the lysozyme isolated after the modification procedure remains active in a cell lysis assay.

The stability of the flavin–lysozyme link is most consistent with that of an ester bond, the type of bond expected from reaction between a carboxylate and an α-bromoacetyl compound. Less than 15% of either flavolysozymes I or II released its cofactor after 11 months at 4 °C, pH 5–7, yet at ambient temperature and pH 9, the cofactor is released with or without added hydroxylamine within 2 h. Seryl, histidyl, and lysyl alkylation products are not likely to be this sensitive to pH.

The pH profile for the modification of lysozyme (Figure 1) is also consistent with carboxylate nucleophiles reacting with the electrophilic α-bromoacetyl species to produce semisynthetic isozymes I and II. Total protein alkylation is clearly facilitated by the deprotonation of a weak acid(s) with a pK_a of ca. 5, a value within the range of that for the β-carboxylate of aspartyl residues in lysozyme.[6] Figure 1 indicates more than just a crucial pK_a; it also delineates the relative reactivity of the surface and active-site nucleophiles that are responsible for the formation of isozymes I and II, respectively. The carboxylate of Asp-52, the active-site Asp, was previously shown to have a pK_a as low as 3.5,[6] lower than the other reactive carboxylates in lysozyme. Therefore, Asp-52 should react with **3b** to form 8-flavolysozyme II preferentially over isozyme I as the pH is lowered from 5 to 3. Figure 1 illustrates that isozyme II in fact becomes the major flavoprotein product as the pH of the alkylation reaction is lowered, a result that supports the proposed structure for flavolysozyme II as the active-site-modified lysozyme.

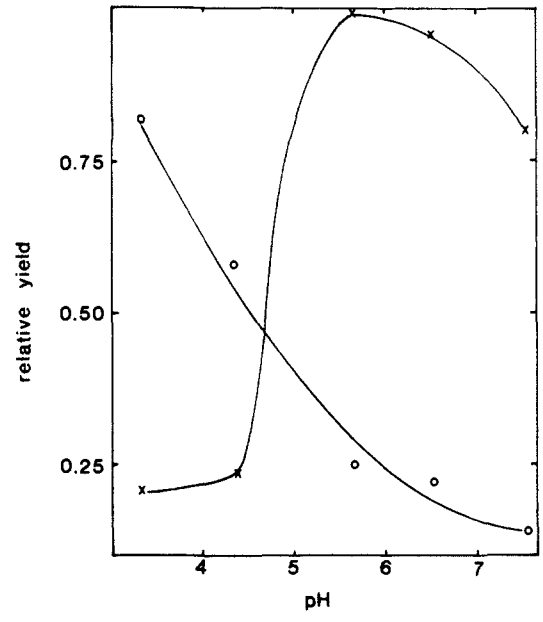

Figure 1. pH profile of lysozyme alkylation by 8α-(bromoacetyl)-10-methylisoalloxazine. Lysozyme (10 mg) in 9 mL of 100 mM potassium phosphate at the indicated pH values was incubated at 60 °C while a total of 5 equiv of **3b** in a minimum of dimethylformamide was added in four aliquots over 4 h. The reaction mixture was then analyzed on a BioRex cation-exchange column (see text). × indicates the sum of 8-flavolysozymes I and II produced at various pH values normalized to the total flavoprotein produced at pH 5.5. The yield of protein alkylation decreases at high pH due to a competing hydrolysis of **3b**. ○ indicates the fraction of 8-flavolysozyme II (relative to the sum of 8-flavolysozyme I and 8-flavolysozyme II) produced at each pH value.

Oxidoreductase Activity of Flavopapain vs. Flavolysozymes I and II in the Presence of Hydrophobic Substrates. Flavopapain efficiently catalyzes the oxidation of dihydronicotinamides designed to bind in the active site of papain.[1] *N*-Propyl-, *N*-hexyl-, and *N*-benzyldihydronicotinamides all seem to bind the hydrophobic groove of papain, allowing the nicotinamide ring easy access to the bound flavin analogue. Since lysozyme possesses an active site quite distinct from that of papain, the low activity of flavolysozymes I and II (produced from **1b**, **2b**, and **3b**) with these substrates was anticipated. When these hydrophobic compounds are used as substrates, the second-order rate constants (k_2) of cofactor-dependent oxidation are equivalent for flavolysozyme and the appropriate free flavin analogue (600–1000 M^{-1} s^{-1}). In addition, these substrates do not detectably form a Michaelis complex with flavolysozyme, and, thus, this semisynthetic enzyme is devoid of flavopapain specificity. A comparison of these two catalysts reveals that the catalytic potential of flavopapain is truly dictated by papain's binding specificity and not merely due to a potential isoalloxazine–dihydronicotinamide complexation common to all flavin-based catalysts.

Oxidoreductase Activity of Flavolysozymes I and II in the Presence of Hydrophilic Substrates. The future utility of flavolysozyme is not precluded by its low activity with the *N*-alkyldihydronicotinamides. The amino acid side chains of lysozyme do not particularly facilitate the above reaction, but neither do they mask the redox activity of the covalently attached cofactor. Thus, the bound flavin should be free to react with a variety of substrates that have an affinity for lysozyme greater than that demonstrated with the *N*-alkyldihydronicotinamides.

One of the attributes of lysozyme that makes it an attractive candidate for a semisynthetic enzyme is its ability to bind a diverse set of such compounds as cytochromes, flavonoids, penicillin, Co^{2+}, Cu^{2+}, and Mn^{2+}.[6] Lysozyme's natural substrate is an alternating polymer of *N*-acetylglucosamine/*N*-acetylmuramic acid, but it will bind many sugars (*N*-acetylglucosamine, glucose, γ- and δ-gluconolactone, 2-deoxyglucose, ribose, etc.[6]) in its large active-site crevice. Readily available derivatives of *N*-ribodihydronicotinamide may then serve as the appropriate substrates for flavolysozyme catalysis.

The catalytic effect of both isozymes of flavolysozyme synthesized from **1b**, **2b**, and **3b** were surveyed with the series of substrates, *N*-ribodihydronicotinamide, NMNH, NADH, and NADPH and compared to the activity of the appropriate free isoalloxazine model. In almost every case, the flavoprotein is more active as an oxidoreductase than is the free cofactor, and the active-site modified enzyme, isozyme II, is more active than the surface-modified enzyme, isozyme I (for example, see data for 8-flavolysozymes I and II in Table I). In addition, the k_2(6-flavolysozyme)/k_2(model flavin) values range from 0.7 to 5 and k_2(7-flavolysozyme)/k_2(model flavin) values range from 1 to 3 in the presence of the above dihydronicotinamides. By a survey

of the activity of the lysozyme-based oxidoreductases with progressively larger substrates, a truly enzymatic catalyst has been discovered. 8-Flavolysozyme II catalyzes the oxidation of NADPH in a saturable manner with the template, lysozyme, enhancing the isoalloxazine-dependent activity 10-fold over the control rate. For a newly developed semisynthetic enzyme such as flavolysozyme, these properties are sufficient to encourage future development of this system.

Except for the reaction between 8-flavolysozyme II and NADPH, saturation kinetics are not detected with the other flavolysozymes in the presence of up to 1.5 mM substrate. However, the relative catalytic activities of 8-flavolysozymes I and II (Table I) still are consistent with the known binding properties of lysozyme. When a flavin analogue is placed within lysozyme's active site (8-flavolysozyme II), productive binding between the substrate and cofactor is more favorable than when this cofactor is placed on the protein surface, near the outer edge of the active site (8-flavolysozyme I). A model of 8-flavolysozyme II suggests that this protein may bind substrates at either end of its large active-site cleft, whereas 8-flavolysozyme I can utilize only one end of the active site to orient substrates.

A second trend of substrate selectivity is shared by both 8-flavolysozymes I and II. The relative rate enhancements for both oxidoreductases increase as the substrate increases in size and negative charge. Larger substrates (e.g., NADH and NADPH) might then utilize more of the binding potential of lysozyme, and substrates with more negative charge may coordinate more efficiently to the very basic lysozyme (p*I* = ca. 11[6]).

General Catalytic and Physical Characteristics of Flavolysozyme. The catalytic power of 8-flavolysozyme II, the most active lysozyme-based semisynthetic enzyme, is reminiscent of that published for the initial papain-based semisynthetic enzymes.[9] Before a second-generation flavolysozyme can be designed to make best use of lysozyme's physical properties, characterization of the current set of flavolysozymes must be extended beyond measuring the above kinetic parameters.

As shown below, the rate-determining step of flavolysozyme catalysis, the chemical step that must be expedited in future flavolysozyme constructs, is the initial transfer of a hydride equivalent from the dihydropyrimidine ring of NADH to the isoalloxazine ring system of the flavoprotein. The reduced form of flavolysozyme does not visibly accumulate during steady-state turnover of the ribose-based dihydronicotinamide derivatives studied here. In addition, the concentration of the ultimate electron acceptor, either O_2 or MTT, used under turnover conditions does not affect the overall rate of catalysis. Consequently, the reoxidation of the flavin must be faster than the initial reduction. Consistent with this rate-determining step, 8-flavolysozymes I and II process [4,4-$^2H_2$]NADH (3.5- and 3.8-fold, respectively) more slowly than the equivalent proteo compound.

Although the substrate specificity of flavolysozyme is set by the template, lysozyme, the general reactivity of this system can be modulated by specific protein–isoalloxazine interactions. These interactions could certainly play a role in determining the greater catalytic activity of 8-flavolysozyme over that of 6- or 7-flavolysozyme. However, the extent to which these interactions are utilized in a given semisynthetic enzyme can be more fully described by studying flavin-dependent reactions that necessitate cofactor–protein interactions.

Four properties characteristic of many naturally occurring flavoproteins[20,21] were used to initially screen flavolysozyme for productive interactions between the covalently bound flavin derivative (at Asp-52 and Asp-101) and lysozyme. Most flavoenzymes stabilize a one-electron-reduced form of the flavin referred to as the semiquinone form. This stabilization may arise from an enzyme's kinetic or thermodynamic control over the flavin.[20] When 8-flavolysozymes I and II were subjected to the usual photoreduction conditions necessary for generating the semiquinone, no characteristic semiquinone spectra were obtained. The photoreduction of 8-flavolysozymes I and II proceeded directly from the oxidized to the two-electron-reduced form.

A second type of flavin modification, flavin–sulfite complexation, was also not stabilized by the environment lysozyme provides for the isoalloxazine ring system of 8-flavolysozyme. The K_D of the control flavin derivative–sulfite adduct (ca. 26 mM) was comparable to that for 8-flavolysozyme II (K_D = 28 mM). This type of adduct was even destabilized for 8-flavolysozyme I (K_D = 230 mM). Natural flavoproteins that function physiologically as a dehydrogenase/oxidase exhibit sulfite–flavin dissociation constants as low as 4 μM.[14]

Finally, many natural flavoproteins display a circular dichroic spectrum (CD) in the visible region[21] and an absorption spectrum that differs from free flavin. The isoalloxazine moiety, although not chiral, will absorb different amounts of left and right circularly polarized light when it is sequestered into a chiral environment such as a protein cleft. 8-Flavolysozyme II, however, had an undetectable visible CD spectrum, indicating that this isoalloxazine derivative was not interacting strongly with the chiral lysozyme active site. In addition, no perturbation of the absorption spectrum of flavin was detected after covalent modification of lysozyme. Therefore, the catalytic power of flavolysozyme cannot be ascribed to specific interactions between the cofactor and protein. The rate enhancements documented thus far might only arise from lysozyme's ability to orient the substrates approach to the bound flavin derivative.

Conclusion

The use of lysozyme as a protein template for semisynthetic enzymes signifies a radical departure from earlier work with the papain template. Lysozyme is a small hydrophilic protein that does not contain a single easily modified nucleophile, yet we were able to synthesize and purify homogeneous species of flavolysozyme. Therefore, template choices in the design of new semisynthetic enzymes may be extended to other proteins that lack a highly reactive amino acid. Flavin derivatives can successfully be attached to proteins through residues other than the usual cysteine thiol. The α-bromoacetyl compounds, **1b**, **2b**, and **3b**, appear to modify lysozyme through a stable ester. In addition, proteins with more than just one active nucleophile can now be considered as candidates in the design of novel semisynthetic enzymes since synthetic isozymes may be separable by chromatography.

Nonspecific hydrophobic interactions were sufficient for coordinating flavopapain's substrates in an aqueous media. However, a new guideline must be considered as semisynthetic enzymes are developed to process hydrophilic compounds operating in an aqueous environment. Very specific protein–substrate and/or protein–flavin interactions will be necessary to orient the reductant and the isoalloxazine derivative for significant rate enhancements.[3]

Flavolysozyme is now the third in a series of semisynthetic enzymes that coordinates substrates in a manner predicted from the published properties of its protein template. From the characterization of the flavolysozymes presented here, a second generation of flavolysozymes may be constructed to utilize further the binding potential of lysozyme, the first template available on a large industrial scale.

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Registry No. 1b, 101916-86-3; **2b**, 68973-54-6; **3b**, 79127-42-7; NMNH, 4229-56-5; NADH, 58-68-4; NADPH, 53-57-6; ribodihydronicotinamide, 19132-12-8.

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