Generation of New Enzymes via Covalent Modification of Existing Proteins

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I. Introduction

Proteins are versatile molecules for catalyst design. Given the explosive development of molecular biology techniques for gene manipulation and functional selection, genetic approaches for enzyme design are currently in wide use. However, chemical methods provide a means for introducing a diverse range of functionality that does not occur in natural enzymes and cannot be easily incorporated by genetic methods. Thus, chemical modification of proteins remains a valuable tool for protein engineering. This article reviews a variety of approaches in which chemical modification has been used to alter the catalytic properties of existing enzymes to give new catalytic activities to existing enzymes or to impart catalytic activities to proteins normally devoid of enzymatic activity. Since the focus of this review is on the chemistry promoted by these systems, modifications that alter the overall stability or general physical properties of enzymes are not discussed here.

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This article is divided into two main sections. In the first section, modifications of enzymes that result in perturbation of their natural catalytic activity are discussed. These include changes in substrate specificity and the introduction of functionality that permits the regulation of enzyme activity by exogenous ligands. This section also covers early work in the field and describes most of the general strategies and specific chemistry that have been used for derivative proteins. The second section reviews work that focuses on the introduction of new enzyme activities into proteins and enzymes. In this part, the functional properties of the resulting conjugates are emphasized with less discussion of the attachment chemistry. Some of the material covered in this article has been described in earlier reviews, and the reader is encouraged to consult those works for more information.[1−9] Included at the end of this article is a section entitled "New Frontiers" that highlights exciting new chemistry that should be applicable to future semisynthetic design approaches.

II. Modification of Existing Enzyme Activity

A. Atom Replacement

Prior to 1966, chemical modification of enzymes was used primarily to identify specific residues present in enzyme active sites that were involved in substrate binding or chemical catalysis. This changed in 1966 when two seminal papers were published in which the authors described a chemical approach for converting the active site serine-derived hydroxyl group present in subtilisin to a thiol.[10,11] To accomplish this, subtilisin was treated with phenylmethanesulfonyl fluoride to activate the nucleophilic serine residue followed by reaction with thioacetate and subsequent hydrolysis to produce thiolsubtilisin as shown in Figure 1.

Despite the enhanced reactivity of a thiol or thiolate in terms of both nucleophilicity and leaving group ability, thiolsubtilisin proved to have only limited catalytic activity; both groups observed reduced rates of hydrolysis at least 100-fold lower for activated nitrophenyl esters (pNP). Furthermore, no reaction was observed with amide-containing substrates such as tosylarginine methyl ester and glutarylphenylalanine *p*-nitroanilide (pNA).[12] Despite the low activity of these semisynthetic enzymes, these studies proved important because they established the methodology that was used to produce an entire

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family of newer semisynthetic enzymes with novel and interesting catalytic activities.

In subsequent work, Yokosawa et al. expanded the semisynthetic approach described above to include the introduction of a thiol group into a protein that contained disulfide bonds by preparing thioltrypsin.[13] Specifically, they functionalized trypsin from *Streptomyces griseus,* which contains three disulfide bonds, using a procedure similar to that employed in the preparation of thiosubtilisin. The resulting enzyme, thioltrypsin, manifested no detectable (<0.01%) activity for the hydrolysis of amide-containing substrates such as Bz-Arg-pNA. However, as was observed with thiosubtilisin, some esterase activity with active substrates including Cbz-Lys-pNP was retained.

Complementary work by Clark and Lowe examined a variant of papain in which the active site thiol was converted to a hydroxyl group.[14] This was accomplished via the procedure outlined in Figure 2.

The key reaction in this case was the photochemical cleavage of an alkylated form of the enzyme to

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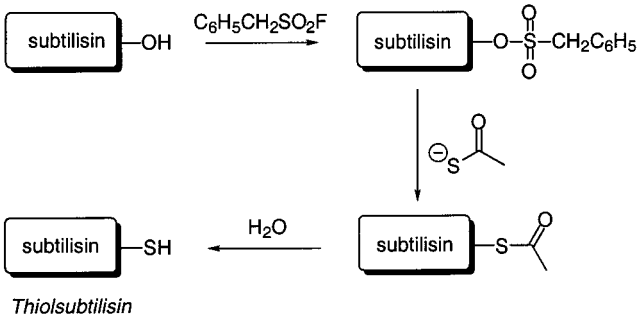


Thiolsubtilisin

Figure 1. Chemical conversion of subtilisin to thiolsubtilisin.

yield a thioaldehyde, which was converted to the desired alcohol by hydrolysis and reduction with $NaBH_4$. Although the authors were able to successfully carry out this atom replacement, an impressive one for the time, the resulting enzyme possessed no

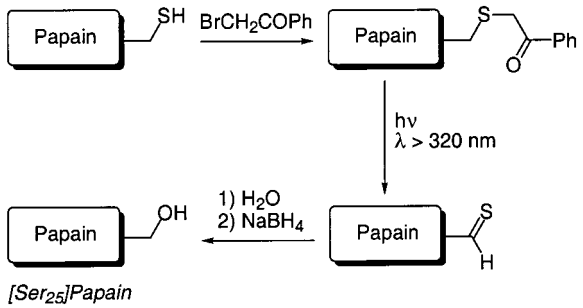


Figure 2. Chemical conversion of the active site thiol in papain to a hydroxyl group.

catalytic activity for even activated ester substrates such as Cbz-Lys-pNP and Cbz-Gly-pNP, which are known to be efficiently hydrolyzed by papain. An emerging conclusion from these atom replacement experiments was that the catalytic machinery of these hydrolytic enzymes was more complex and finely tuned than was generally appreciated.

The initial observation that thiolsubtilisin was unable to hydrolyze amide-containing substrates lead Kaiser and co-workers to consider the idea of exploiting this semisynthetic enzyme to perform amide bond-forming reactions in mixtures of DMF and water.[15] The concept, illustrated in Figure 3, was to

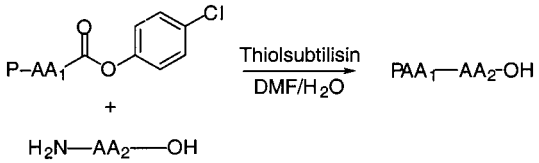


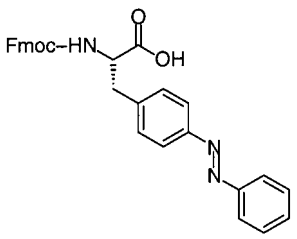
Figure 3. Scheme for the use of thiolsubtilisin as a peptide ligase. In this scheme P is a protecting group, AA_1 is an N-terminal amino acid or peptide component, and AA_2 is a C-terminal amino acid or peptide component.

acylate the active site thiol with an activated ester of an amino acid or peptide and then capture the resulting thioester intermediate with a second amino acid or peptide possessing a free amino terminal nucleophile. The investigators examined a number of amino acid donors and acceptors and found high levels of conversion in many cases. Interesting observations included the following: (a) the ligation reaction was stereospecific, with an absolute preference for the L-isomers; (b) protection of functional groups including serine and tyrosine hydroxyls and the C-terminal carboxyl group of the acyl acceptor was unnecessary; (c) the substrate specificity was quite broad; and (d) the enzyme was catalytic, performing up to 68 turnovers. The authors were also able to prepare several peptides including [Leu]^5 enkepalin amide and RNase T1 (12−23). Interestingly, the latter product was obtained by thiolsubtilisin-catalyzed condensation of an activated octameric peptide and cognate tetramer. Thus, both simple dipeptides as well as more complex polypeptides could be assembled using this versatile semisynthetic catalyst. It is interesting to note that these results obtained with thiolsubtilisin produced via chemical methods paved the way for more recent work by Wells and co-workers in which forms of thiolsubtilisin termed "subtiligases" with improved properties were produced by genetic methods.[16−23] Thiolsubtilisin has also been examined in a variety of other less polar organic solvents. In water, subtilisin is 30-fold more efficient than thiolsubtilisin at hydrolyzing an activated ester substrate, pNPB. However, in organic media such as hexane, dioxane, and acetonitrile, subtilisin is only 5- to 6-fold more efficient.[24]

B. Segment Reassembly

An alternative approach to atom replacement for chemical modification of enzyme activity involves the replacement of large portions of a protein via selective proteolysis or chemical cleavage followed by separation of the resulting fragments and reconstitution with a chemically modified fragment. In some cases it is possible to reform the scissile peptide bond, while in others it is not. Interestingly, in some situations it is not necessary to effect amide bond reformation to obtain catalytic activity. Ribonuclease A (RNase A) is a particularly useful example of this. Native RNase A can be cleaved using subtilisin to yield two tightly associated fragments denoted as the S-peptide (residues 1−20) and the S-protein (residues 21−124). While neither of these fragments displays observable catalytic activity, the complex denoted as RNase S retains the native structure and full catalytic activity of RNase A. Of particular utility is the fact that this noncovalent complex can be denatured and separated and the resulting purified S-protein renatured in the presence of a modified S-peptide. Peptides as short as 15 amino acids containing residues 1−15 of RNase A can be complexed with S-protein to produce a complex with altered properties. This useful feature has resulted in RNase S being used as the basis for a plethora of semisynthetic constructs.

Since the S-peptide of RNase S is required for catalytic activity, modifications of this peptide can modulate the rate of enzymatic reaction; such systems could form the basis of a new generation of molecular sensors. An ingenious construct based on this idea was reported by Shinkai and co-workers in which a photosensitive amino acid, phenylazophenylalanine (Z), was incorporated into the S-peptide at several positions as shown in Figure 4.[25]

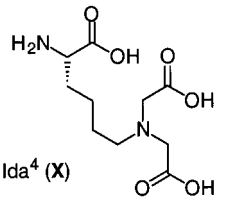


Fmoc-Phenylazophenylalanine (**Z**)

```
                1       8       15
4-1 WT          KETAAAKFERQHODS
4-2 MUT1       ZKETAAAKFERQHODS
4-3 MUT2        KETAAAKZERQHODS
4-4 MUT3        KETAAAKFERQHZDS
4-5 MUT4        KETAAAKFERQHODSZT
```

Figure 4. Phenylazophenylalanine and its incorporation into RNase S-peptides. The phenylazophenylalanine residues are represented by Z. Norleucine is indicated by O.

When the peptides were initially reconstituted with S-protein, a variety of effects were seen. In one case (**4**−**2**), wild-type activity was observed in both the *cis*- and *trans*-azo isomers. In another case (**4**−**3**), very little activity was seen with either of the isomeric forms. However, in one case, the hybrid RNase S′ (**4**−**4**) displayed significant activity in the *trans* form and no observable activity when photoisomerized to the *cis* isomer. As such, this construct provides an interesting example of how light can be transduced to an enzymatic signal. The last hybrid RNase S′ (**4**−**5**) was slightly more active in the *cis* form than in the *trans* form. In related work, Hamachi et al. prepared forms of RNase S that are modulated by metal ions and polyamines.[26,27] In the former, iminodiacetic acid (Ida) moieties were introduced into the S-peptide at various locations (**5**−**1** through **5**−**4**), as noted in Figure 5, and the resulting S-peptides

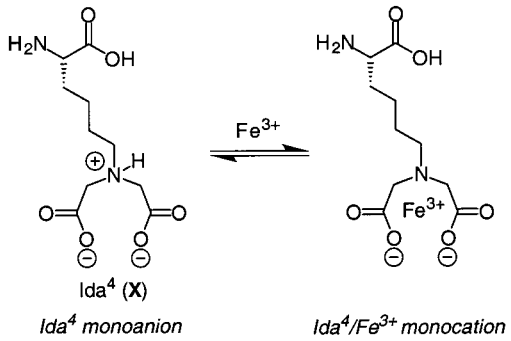


		1 8 15
	WT	KETAAAKFERQHMDS
5-1	T3X	KE**X**AAAKFERQHMDS
5-2	A6X	KETAA**X**KFERQHMDS
5-3	E9X	KETAAAKF**X**RQHMDS
5-4	D14X	KETAAAKFERQHM**X**S
5-5	T3X,A6X	KE**X**AA**X**KFERQHMDS
5-6	A6X,E9X	KETAA**X**KF**X**RQHMDS
5-7	E9X,D14X	KETAAAKF**X**RQHM**X**S
5-8	T3X,E9X	KE**X**AAAKF**X**RQHMDS
5-9	A5X,E9X	KETA**A**AKF**X**RQHMDS

Figure 5. Amino acid containing iminodiacetic acid (Ida[4]) and its incorporation into RNase S-peptides. The Ida[4] residues are represented by X.

were reconstituted with S-protein. Using CD spectroscopy, they showed that the amount of S-peptide α helicity in the presence of Cu(II) varied depending on the precise location of the Ida residue. A variety of other divalent metals including Zn(II), Co(II), and Ni(II) showed very little effect. In peptides containing two Ida residues (**5**−**5** through **5**−**9**), both 1:1 peptide/Cu(II) and 1:2 peptide/Cu(II) ratios were observed. Enzyme activity correlated with peptide helicity in the presence of Cu(II). These observations lead the investigators to propose a dual-mode model in which the activity of RNase S could be suppressed or enhanced depending on the precise concentration of Cu(II). Hamachi and co-workers extended their work with Ida-containing S-peptides by preparing a series that displayed the Ida moiety at several positions (**6**−**1** through **6**−**6**) located on the surface normally occupied by cationic or anionic residues in the wild-type S-peptide as illustrated in Figure 6.[28]

Reconstituted RNase S′ enzymes incorporating these dianionic Ida residues varied in their activity. In cases where Ida was introduced into a position formerly bearing an anionic residue (**6**−**2**, **6**−**4**, **6**−**6**), the resulting enzyme activity was comparable to the RNase S. In contrast, incorporation of Ida into



		1 8 15
	WT	KETAAAKFERQHODS
6-1	K1X	**X**ETAAAKFERQHODS
6-2	E2X	K**X**TAAAKFERQHODS
6-3	K7X	KETAAA**X**FERQHODS
6-4	E9X	KETAAAKF**X**RQHODS
6-5	R10X	KETAAAKFE**X**QHODS
6-6	D14X	KETAAAKFERQHO**X**S
6-7	A6X,E9X	KETAA**X**KF**X**RQHODS
6-8	K7X,R10X	KETAAA**X**FE**X**QHODS

Figure 6. Peptides containing an iminoacetic acid moiety for metal-induced Umpolung. The Ida[4] residues are represented by X. Norleucine is indicated by O.

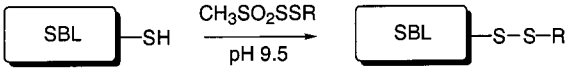
positions formerly bearing cationic residues (**6**−**1**, **6**−**3**, **6**−**5**) lead to decreases in activity. Most interestingly, the activities of these semisynthetic complexes could be modulated by the addition of Fe(III), which inverted the charge at the Ida-containing position from a dianion to a monocation. Thus, in the presence of Fe(III), complexes containing Ida at formerly cationic positions (**6**−**1**, **6**−**3**, **6**−**5**) increased in catalytic activity while Ida at positions formerly bearing anionic residues (**6**−**2**, **6**−**4**, **6**−**6**) decreased enzyme activity. The authors termed this inversion in relative activity "guest-induced umpolung on a protein surface". Further work showed that a double mutant (7,10-Ida4-RNase S′) containing Ida residues at two formerly cationic positions (**6**−**8**) gave a more dramatic response upon addition of Fe(III). In the absence of Fe(III), 7,10-Ida4-RNase S′ manifested only low levels of enzyme activity. However, upon addition of Fe(III), this new construct displayed a 20-fold increase to wild-type levels of hydrolytic activity. Interestingly, this rise in activity was mirrored by an increase in α helicity of the protein, suggesting that the charge inversion (umploung) mediated by Fe(III) was responsible for returning the modified S-peptide to a wild-type-like conformation.

C. Specificity Modification

In principle, it should be possible to use chemical modification of enzymes to alter their substrate specificity. Unfortunately, applying this concept to real enzymes is complicated by the polyfunctional nature of proteins. In general, selective funtionalization of a single residue in a protein is quite difficult. Of the various functional groups present in proteins, thiols are the most amenable to specific modification. This is a combination of the fact that cysteine, the only thiol-containing residue, is less common than many other amino acids and that thiols can be selectively modified in the presence of other less

potent nucleophiles present in proteins. Modification can be accomplished either by selective alkylation with α-halocarbonyl-containing compounds or malaimides or by disulfide bond formation. These features have become particularly attractive in light of the development of site specific mutagenesis methods that allow new cysteine residues to be introduced or existing ones to be deleted at specific sites within an enzyme. Essentially, enzymes can be engineered to have only one cysteine residue accessible for modification, which in turn can be used to introduce a wide variety of new functionality into enzymes. Here, several examples of how the substrate specificity of enzymes can be altered using this two-step approach of mutagenesis and chemical modification are described.

Subtilisins are an important class of proteases used in a number of synthetic and industrial applications. Hence, the ability to tailor the specificity of these enzymes is highly desirable. Jones and co-workers made useful progress toward attaining this goal using a combination of site-directed mutagenesis and chemical modification. On the basis of inspection of the X-ray structure of subtilisin *Bacilus lentus* (SBL), two positions, N_{62} and L_{217}, in the S_2 and S_1' pockets, respectively, were mutated to cysteine residues. These cysteine-containing mutants were modified with a series of methanethiosulfonates containing a variety of substituents with a range of sizes, shapes, and charges as noted in Figure 7.[29,30]



7-1: R = CH_3
7-2: R = CH_3CH_2
7-3: R = $(CH_3)_2CHCH_2$
7-4: R = $CH_2C(CH_3)_3$
7-5: R = $(CH2)_4CH_3$
7-6: R = $(CH2)_5CH_3$
7-7: R = $(CH2)_9CH_3$
7-8: R = CH_2-*c*-C_6H_{11}
7-9: R = $CH_2C_6H_5$
7-10: R = $CH_2CH_2SO_3^-$
7-11: R = $CH_2CH_2NH_3^+$

Figure 7. Methanethiosulfonate-based reagents for the modulation of subtilisin activity.

Interestingly, although the introduction of the cysteine mutations initially resulted in a decrease in catalytic efficiency, the subsequent chemical modifications reversed this effect and in some cases produced enzymes with efficiencies (k_{cat}/K_M) greater than that of wild type. Using a similar approach, Jones and co-workers prepared additional mutations in the S_1 and S_1' pockets of SBL. Modification of the cysteine $M_{222}C$, a buried residue in the S_1' pocket in SBL, with a series of methanethiosulfonates resulted in significant decreases in catalytic efficiency to levels, as much as 122-fold lower than wild type.[31] More modest activity changes ensued upon modification of $S_{166}C$, a position within the S1 pocket. Interestingly, introduction of a positive charge (**7**−**11**) into this protein had no effect on k_{cat}/K_M, while the introduction of a negative charge (**7**−**10**) lowered activity. Modification of a third mutant enzyme, $S_{156}C$, with the same ensemble of reagents produced only minimal changes in activity. These latter results were not surprising given that S_{156} in the wild-type structure is exposed on the protein surface with the hydroxyl group solvated. Molecular modeling of the chemically modified forms of the $S_{166}C$ mutant proteins suggested an interesting structure−activity relationship with the β-carbon of Cys_{166}. Model building and molecular mechanics analysis illustrated that k_{cat}/K_M for the chemically modified proteins correlates with the extent of binding distortion of the P1 benzyl side chain conformation of a product inhibitor, AAPF; k_{cat}/K_M decreases as the β-carbon of Cys_{166} points further into the P1 pocket and as the inhibitor side chain is distorted from the conformation it adopts when complexed with the wild-type protein.

The above results with chemically modified cysteine mutants of SBL established the power of that approach and provided the framework for future extensions in which specific enzyme properties were targeted for alteration. An exciting example was the use of chemical modification of $S_{166}C$ to obtain enzymes with different specificity properties.[32] The methanesulfonate-based reagents for this application are shown in Figure 8.

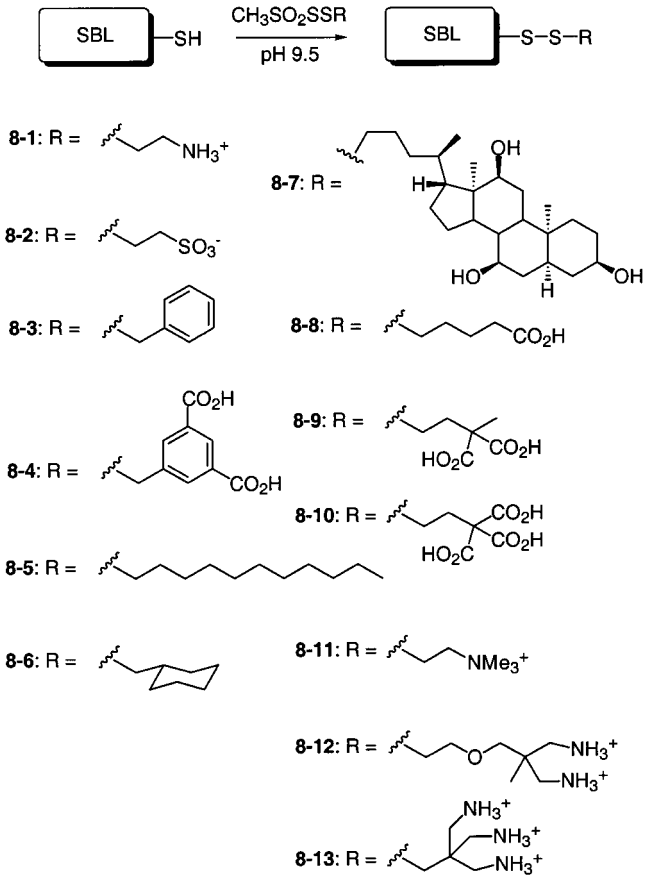


Figure 8. Reagents used to obtain subtilisins with altered specificity properties.

Wild-type SBL shows a marked preference for substrates with large hydrophobic P1 residues. To alter this, the S1 pocket of SBL was filled in by modification of $S_{166}C$ with a bulky cyclohexyl group (**8**−**6**). This lead to a 2-fold increase in k_{cat}/K_M for a

suc-AAPA-pNA substrate and a 51-fold improvement in selectivity for suc-AAPA-pNA versus suc-AAPF-pNA compared to wild-type SBL. The ability of SBL to tolerate multiple negative charges within the substrate-binding pockets was examined in a series of mutants at positions 62 (S_2 site), 156 (S_1 site), 166 (S_1 site), and 217 (S_1').[33] In each case, cysteine was introduced into these positions and modified with the anion-containing reagents (**8**−**4**, **8**−**8**, through **8**−**10**) shown in Figure 8. The presence of up to three negative charges in the various SBL subsites resulted in up to 11-fold decreased activity using a normal SBL substrate, suc-AAPF-pNA. The mutant at position 166 was chosen for additional studies which examined the effects of anionic chemical substitutions (**8**−**8** through **8**−**10**) on the hydrolysis of a charge-complementary cationic substrate.[34] The resulting constructs showed up to 9-fold improvements in k_{cat}/K_M for the substrate su-AAPR-pNA and a concomitant 61-fold improvement in suc-AAPR-pNA/suc-AAPF-pNA selectivity. Using a similar concept for promoting anionic substrate binding, a conjugate bearing an aminoethyl modification (**8**−**1**) showed a 19-fold improvement in k_{cat}/K_M for suc-AAPE-pNA and a 54-fold improvement in suc-AAPE-pNA/suc-AAPF-pNA selectivity compared to wild-type SBL. Substitutions at positions 62, 156, 166, and 217 with a series of mono-, di-, and tricationic groups (**8**−**1**, **8**−**11**, through **8**−**13**) allowed the effect of multiple positive charges in the S_1, S_2, and S_1' sites to be investigated.[35] These modifications lead to a general diminution in catalytic efficiency of as much as 77-fold, possibly due to perturbation of the protonation state of the histidine residue from the catalytic triad critical for enzymatic activity.

Jones and co-workers also examined the ability of chemically modified forms of SBL to modulate the ratio of esterase to amidase activity;[36,37] such modified enzymes could have valuable properties for a host of applications. Using $N_{62}C$, $L_{217}C$, $S_{166}C$, and $M_{222}C$ mutant forms of SBL and subsequent derivatization with a series of methanethiosulfonate reagents, a library with approximately 40 members was prepared. Esterase and amidase activities were evaluated initially in a high-throughput microtiter plate-based screen; 25 of these modified proteins were chosen for more complete kinetic analysis using suc-AAPF-pNA (amidase substrate) and suc-AAPF−SBn (esterase substrate). Of these 25 constructs, 20 displayed improved esterase to amidase activity up to 52-fold. Additionally, 19 manifested esterase activity that was as high as 3-fold higher than wild-type SBL. While the specific explanations for all the rate perturbations observed are not known, it is clear that through screening libraries of chemically modified proteins, unexpected and interesting catalytic properties can be discovered. Finally, other aspects beyond enzyme specificity and rate can be tuned using the chemical modification approach. Preparation of a series of disulfide-linked conjugates (**7**−**1** through **7**−**11**) based on a $N_{62}C$ mutation in SBL lead to the observation that the rate versus pH profile for the enzyme-catalyzed reaction could be perturbed accompanied by significant (2-fold) increases in k_{cat}/K_M.[9,34] On the basis of literature precedent, the authors ascribed the pH effect on k_{cat}/K_M as a reflection of the pK_a of His_{64}, a component of the catalytic triad. Intriguingly, a linear correlation between the pK_a of this residue and the log P of the group introduced by chemical modification was noted. Modeling experiments suggested that modifications of $N_{62}C$ would perturb the hydrophobicity around His_{64}, consistent with the observed shift in pK_a. When viewed together, these chemical modification experiments exemplify a versatile approach for the modulation of enzymatic activity. While the reagents described are relatively modest in the range of functionality that they introduce, there is no reason such a strategy could not be used for the introduction of more complex functionality including various heterocyclic systems. While this has not yet been done with a specific protein, Viola and co-workers developed a series of reagents designed to allow a variety of nitrogen-containing heterocycles to be linked to protein-derived cysteine residues.[38]

An ingenious means of introducing elements for highly specific recognition via hydrogen bonding was introduced by Schultz and co-workers in their design of semisynthetic DNA-cleaving enzymes.[39] Their approach began with a mutant form of staphylococcal nuclease ($K_{116}C$) that incorporated a unique cysteine residue near the active site. This enzyme cleaves DNA with minimal sequence specificity. However, upon derivatization of the protein with an oligonucleotide of defined sequence 15 bases in length via formation of a disulfide bond (see Figure 9), a

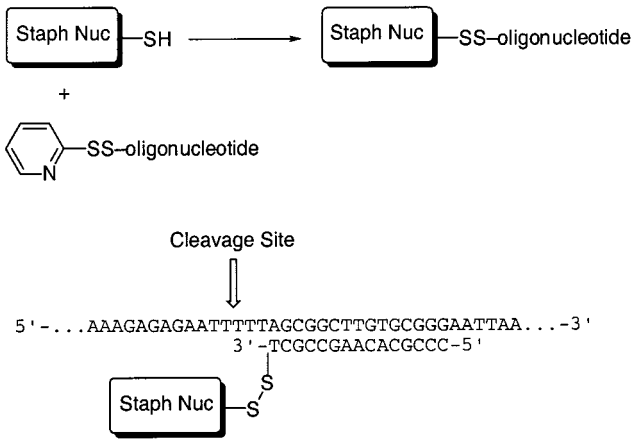


Figure 9. Conversion of staphylococcal nuclease to a sequence-specific nuclease.

significant change in specificity occurs. The resulting conjugate cleaved a single-stranded DNA segment 64 nucleotides in length almost exclusively at one site. The presence of neighboring minor cleavage sites likely resulted from the flexibility of the six methylene spacer that linked the 3′ nucleotide of the oligonucleotide to C_{116}. In complementary work, these investigators showed that they could extend the specificity of this semisynthetic enzyme to include the cleavage of single-stranded RNA.[40] In this case, the same $K_{116}C$ mutant was linked to a 14 base deoxyribonucleotide. The resulting conjugate cleaved a 59 base segment of RNA after hybridization and activation with Ca^{2+} over a 3−5 nucleotide region directly

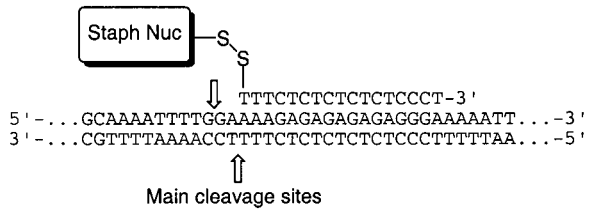


Figure 10. Preparation of a semisynthetic form of staphylococcal nuclease that cleaves double-stranded DNA via triple-helix formation.

adjacent to the hybridization site. Compared to the cleavage of the DNA substrate, hydrolysis of the RNA substrate occurred over a broader region in the target. Cleavage of RNA by this catalyst was extended to larger, naturally occurring pieces of RNA including *E. coli* M1 RNA (377 bases), 16 S rRNA (1542 bases), and yeast tRNAPhe.[41] In the case of M1 RNA, the hydrolysis occurred predominantly at one phosphodiester bond. Importantly, the products of these hydrolysis reactions could be religated to produce the original, full-length RNAs. The above experiments demonstrated that highly selective nucleases with specificities that can be tuned by simple alteration of the oligonucleotide component can be prepared from staphylococcal nuclease. Cleavage reactions proceeded to high extents of conversion in many cases. However, such conversion was only possible using excess enzyme. Thus, these semisynthetic enzymes were not true catalysts. To address this issue, Schultz and co-workers made two changes to their design approach.[42] First, they worked to decrease the nonspecific background cleavage rate by decreasing k_{cat}/K_M for the oligonucleotide-independent cleavage reaction. This was accomplished by introducing two additional mutations, $Y_{113}A$ and $L_{37}A$, into the $K_{116}C$ mutant used in the earlier experiments. These residues had previously been implicated in forming specific interactions with the enzyme substrate. Additionally, the tether length between the enzyme-derived cysteine residue (C_{116}) and the oligonucleotide was shortened to decrease the amount of flexibility in the resulting conjugate. Excitingly, a conjugate incorporating these modifications cleaved a 78 nucleotide DNA substrate, catalytically, when performed at elevated temperatures near the T_m of the oligonucleotide−substrate complex. Using a 1:200 nuclease/substrate ratio, employing a 78 nucleotide substrate, a K_M of 120 nM and k_{cat} of 1.2 min^{-1} were obtained; approximately 30 turnovers of this substrate were observed in 30 min.

While all the original studies were performed on single-stranded DNA or RNA substrates, Schultz and co-workers also adapted their semisynthetic nuclease approach to permit the cleavage of double-stranded targets. To accomplish this, a polypyrimidine-containing oligonucleotide was appended to a mutant of staphylococcal nuclease ($K_{84}C$). The oligonucleotide recognition element was designed to hybridize to double-stranded DNA via triple-helix formation through Hoogsteen hydrogen bonding as shown in Figure 10.[43] Upon activation with Ca^{2+}, the resulting construct cleaved a 70 base pair double-stranded DNA fragment adjacent to the 5′ side of the homopurine target. Microgram amounts of a large plasmid (4433 base pairs) were also cleaved by this nuclease with greater than 75% efficiency at room temperature within 1 h.

An alternative strategy, in lieu of triple-helix formation, was also explored for obtaining double-stranded cleavage products. In this latter approach, applicable to supercoiled plasmid targets, hybridization of the semisynthetic enzyme to the double-stranded DNA was accomplished via D-loop formation facilitated by partial denaturation of the duplex DNA as shown in Figure 11.[44]

Using a nuclease consisting of the staphylococcal nuclease $K_{84}C$ mutant and a 17 or 19 base oligonucleotide, pUC19, a 2686 base pair plasmid, was cleaved at a single site. For this D-looping-based strategy to work, the supercoiled form of DNA was essential; specific cleavage was not observed with the linearized plasmid. Subsequent work by Corey and co-workers demonstrated that cleavage by these nuclease-based constructs was sensitive to local DNA topology displaying greatest efficiencies when target sequences were localized within cruciform regions.[45] The extent of cleavage and specificity depended on the degree of supercoiling in the plasmid target. Interestingly D-looping by these conjugates was not significantly inhibited by a large excess of unlinked oligonucleotide. Thus, elements within both the oligonucleotide and the nuclease itself must contribute to the overall affinity of these constructs for their cognate plasmid target sites. Interestingly, the nuclease component appeared to affect the kinetics of hybridization as well. Experiments that measured the rate of D-loop formation by oligonucleotides alone and a oligonucleotide−staphylococcal nuclease conjugate demonstrated that the presence of the protein moiety increases this association rate by 12 000-fold.[46]

Finally, it is interesting to note that the oligonucleotide−staphylococcal nuclease approach for the design of sequence-specific nucleases has been applied to another enzyme. As noted above, RNase S can be reconstituted with chemically modified forms of the

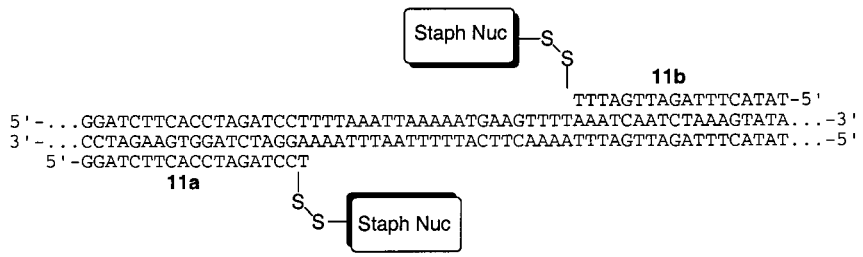


Figure 11. Cleavage of duplex DNA via D-loop formation with a semisynthetic staphylococcal nuclease.

S-peptide. Reconsititution of RNase S-protein with a synthetic S-peptide containing a K_1C mutation functionalized with a disulfide-linked oligonucleotide produced a hybrid RNase S which cleaved RNA adjacent to the oligonucleotide-binding site.[47] Thus, this approach uses a combination of the segment reassembly and specificity modification methods outlined above.

D. Covalent Cofactor Attachment

Enzymes frequently use reactive cofactors in catalysis, although these moieties are generally not covalently bound to the protein. However, covalent attachment can be used to modify the properties of the cofactor or to introduce new cofactors that alter the overall chemistry catalyzed by the enzyme. The attachment of FAD and NAD analogues to modulate existing enzymatic properties is described below. The subject of new cofactors is discussed in a later section in the review. The modification of an apo-flavoenzyme by the covalent attachment of a flavin analogue has been studied recently.[48−50] The most common linkage between protein and flavin is a histidyl(N1 or N3)−(8α)FAD bond. This bond usually forms in an autocatalytic process upon folding of the enzyme inside a cell. Once the bond is formed, it cannot be cleaved without degradation of the protein.[49] Since the apoenzyme is not accessible, it is not possible to study this reaction or incorporate flavin analogues into such enzymes. To accomplish this, an alternative way has been investigated for introducing a protein−FAD bond at the active site of 6-hydroxy-D-nicotine oxidase (6HDNO). This was achieved by replacing the original FAD-binding histidine with a cysteine residue. This allowed the apoprotein to be isolated and alkylated by either 8-chloro-FAD or 8-(methylsulfonyl)-FAD.[50] The enzyme activities of these new conjugates were observed to be approximately 80% of that of the wild-type protein.

More recently, Massey and colleagues used a similar approach to synthesize an artificial flavoenzyme that contained a covalently bound flavin.[48] In this case, G_{281} of human D-amino acid oxidase was replaced with cysteine by site-directed mutagenesis, followed by the attachment of flavin analogue(8-methylsulfonyl FAD) to the free thiol of the engineered cysteine residue. The resulting conjugate displayed a turnover number 26% of that of the wild-type enzyme and was about 40% higher than the turnover number determined for the mutant enzyme preincubated with FAD. However, the K_M value for D-alanine obtained with the mutant enzyme was about 40% lower than that of the covalently linked flavin enzyme. Interestingly, both K_M values of the two mutant proteins, preincubated and covalently linked with flavin, were an order of magnitude higher than that of the wild-type enzyme. These data combined with the results of the limited proteolysis and benzoate-binding experiments showed that the mutant proteins had lower catalytic activity and flavin affinity. This indicated that mutation perturbed the protein structure and catalytic properties, since the mutant protein had a less packed conformation than that of the wild-type enzyme. However, the flavinylation alone did not have a negative impact on the catalytic properties of the protein.

By covalently attaching a FAD analogue to FAD-dependent amino acid oxidase (DAAO), Stocker and co-workers[51] prepared a semisynthetic oxidase, N^6-(6-carboxyhexyl)-FAD-DAAO shown in Figure 12 (**12**−**1**).

This semisynthetic DAAO did not require exogenous FAD and showed very similar kinetic properties to the native holoenzyme, indicating that the chemically introduced FAD moiety in the semisynthetic enzyme bound to a similar position as the free FAD cofactor in the holo DAAO and produced a functional enzyme. Importantly, this semisynthetic enzyme was a considerably more stable enzyme that showed meso-thermostability and withstood inactivation upon dilution.

In a similar approach, a NAD analogue, N^6-[*N*-6-aminohexyl) carbamoylmethyl]−NAD, was tethered to horse liver alcohol dehydrogenase in a carbodiimide-mediated reaction shown in Figure 12 (**12**−**2**).[52] The specific activity of the resulting semisynthetic enzyme was 37−60% of that of native enzyme. What was particularly significant was that the semisynthetic enzyme did not require exogenous NAD and was at least 50 times more efficient than the native enzyme with equimolar free NAD cofactor. Importantly, the tethered NAD cofactor could be regenerated using a second enzyme, lactate or malate dehydrogenase.[53] In a slightly different approach, a Cys residue was introduced on the surface of glucose dehydrogenase (GlcDH) from *Bacillus subtilis* using site-directed mutagenesis and a NAD analogue was incorporated covalently into the enzyme via a disulfide linkage as illustrated in Figure 12 (**12**−**3**).[54] The GlcDH−Cys_{44}−NAD complex functioned together with lactate dehydrogenase in a coupled enzymatic regeneration of NAD(H). L-Lactate and gluconic acid were continuously produced from pyruvate and D-glucose, respectively, with a turnover number of 45 min^{-1} for each NAD molecule. The total turnover number per coenzyme was 125 000 for the first 2.5 days. Therefore, this system had the advantage of converting both NAD and NADP to their more valuable reduced forms at very high recycling rates.

III. Introduction of New Enzyme Activities into Proteins and Enzymes

Covalent modification of enzymes and proteins has been used extensively for the creation of enzymes with new activities. In general, this has been accomplished using the strategies described in the first part of this article. As noted above, the unique reactivity of the thiol functional group derived from cysteine has played a central role in allowing proteins to be functionalized in a site-specific manner. Here, the preparation of new systems that incorporate metal ions via the covalent attachment of ligands is described first. The conjugation of nonmetal-containing cofactors and functional groups with proteins is presented next. Finally, a brief section describing molecular imprinting approaches is included.

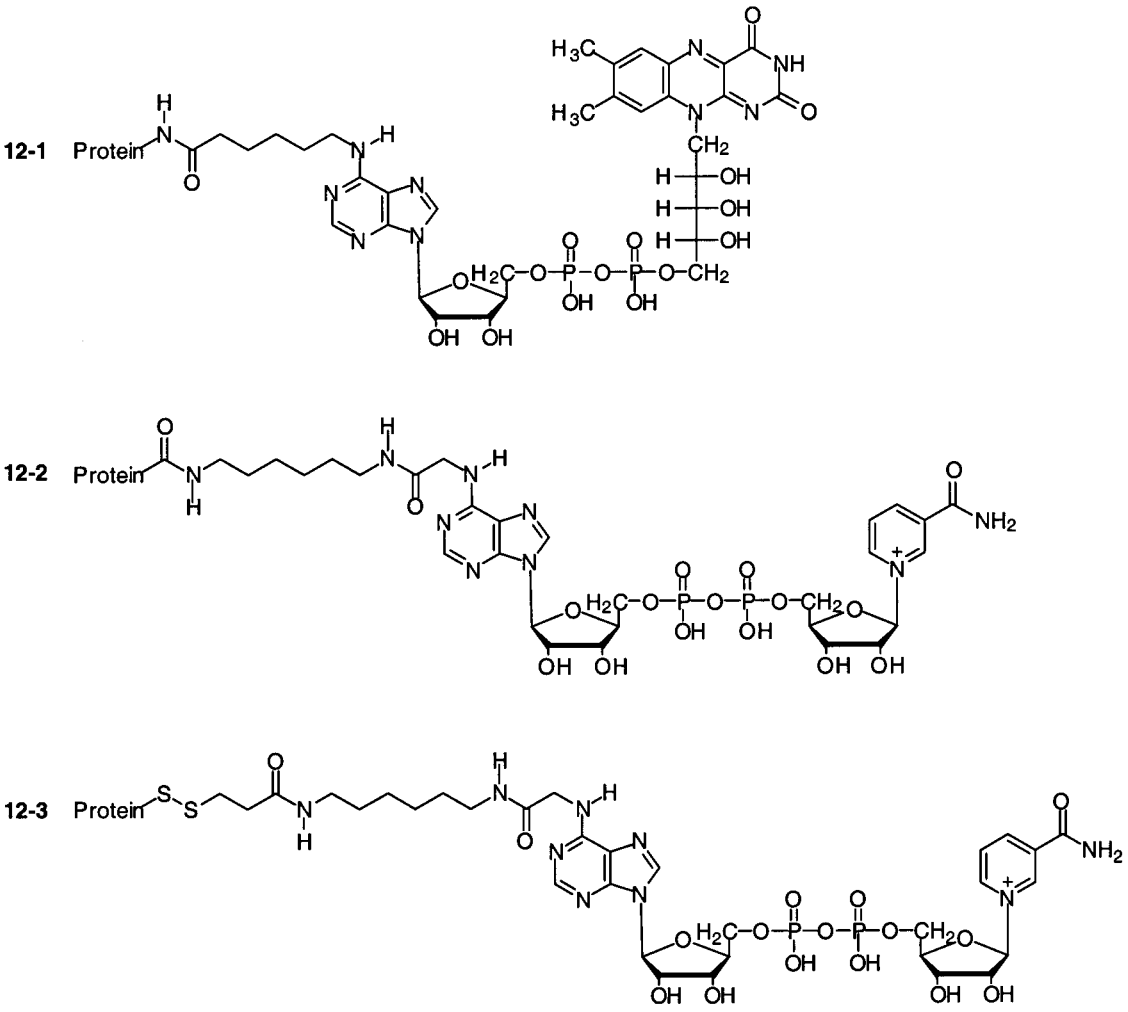


Figure 12. Schematic representation of covalently tethered enzyme−coenzyme complexes: (a) N6-(6-carbamoylhexyl)-FAD-D-amino acid oxidase (DAAO), (b) N6-(6-carbamoylhexyl)−NAD horse liver alcohol dehydrogenase (LADH), (c) glucose dehydrogenase (GlcDH)−NAD complex through a disulfide linkage.

A. Metal-Containing Systems

1. Constructs Containing 1,10-Phenanthroline

The chemical nuclease 1,10-*o*-phenanthroline(OP)−copper is a redox-active coordination complex which cleaves the phosphodiester bonds of nucleic acids to achieve scission of the nucleic acid backbone. Figure 13 illustrates an abbreviated chemical mechanism for the DNA cleavage process. Oxidative attack of a copper−oxo (or copper-bound hydroxyl radical) on the C−1H of the deoxyribose within the minor groove produces the 3′- and 5′-phosphomonoesters, free bases, and 5-methylene furanone (5-MF).[55] Therefore, DNA-binding proteins could be converted into site-specific nucleases by linking them to OP−copper. Minor groove-directed chemistry of OP−copper is particularly advantageous because the contacts between specific target sequence and DNA-binding proteins are primarily in the major groove. Thus, attachment of the chemical cleavage functionality does not disrupt crucial binding determinants.

The first example of a nuclease prepared by combining OP with a DNA-binding protein involved the *E. coli trp* repressor (TrpR),[56] a protein possessing a helix−turn−helix motif interacting in the major groove of the target sequence.[57−59] The two-step

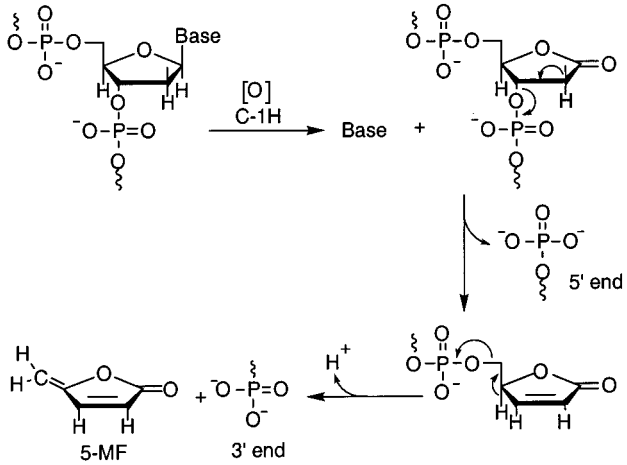


Figure 13. Abbreviated chemical mechanism for the scission of DNA by 1,10-phenanthroline−cuprous complex showing the eventual cleavage products.

modification procedure is summarized in Figure 14.[56,60,61]

The lysines of *E. coli trp* repressor were first modified with 2-iminothiolane hydrochloride in the presence of the corepressor L-tryptophan. The sulfhydryl groups generated by the modification of primary amino groups in the first step were then

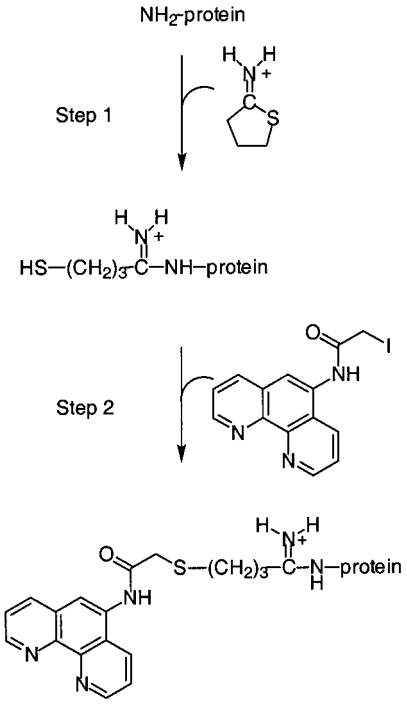


Figure 14. Two-step method for the attachment of the 1,10-phenanthroline moiety to a protein via lysine derivatization.

alkylated with 5-(iodoacetamido)-1,10-phenanthroline. Since *E. coli* TrpR contains four lysyl residues, four OP moieties were incorporated per subunit.

DNase I footprinting assays demonstrated that the *trp* repressor chemically modified by phenanthroline (OP-TrpR) bound to the *aro*H operator site with comparable affinity as the unmodified protein. This indicated that the modification did not interfere with the DNA-binding capability of TrpR. Cutting was dependent on the presence of tryptophan, which serves as a corepressor and is essential for the sequence-specific binding of the *trp* repressor.[61,62] After the initial formation of the OP-TrpR-L-tryptophan complex, the chemistry responsible for strand scission involved multiple steps, including cupric ion coordination to OP, reduction of Cu(II) by exogenous thiols, and reaction with molecular oxygen to produce hydrogen peroxide, which reacts with the cuprous complex to form the copper−oxo species. As noted above, this copper−oxo species is directly responsible for nucleolytic cleavage. Assay of scission on both strands showed a 3′ staggered pattern characteristic of cleavage in the minor groove.[56] Any chemistry that depended on major groove attack would presumably be blocked by the carrier protein. In their initial work, reactions were performed by incubating the *trp*EDCBA operator with OP−TrpR copper complex for 20 h. After incubation, double-strand scission and single-strand nicks in 50% of the labeled strands were observed.[56]

Although an effective scission reagent was generated, it was not clear which 1,10-phenanthroline-modified lysyl residue was most responsible for the nucleolytic activity. This question could be addressed in a general way by introducing cysteine residues at unique positions in DNA-binding proteins genetically and then alkylating them with 5-(iodoacetamido)-1,10-phenanthroline. The first example using mutagenesis and chemical modification to obtain a site-specific scission reagent involved modifying the bacteriophage λ Cro protein.[63] This protein is a homodimeric repressor which provided the first crystal structure of the 'helix−turn−helix' motif.[64] The helix−turn−helix element is responsible for DNA sequence recognition, and five C-terminal residues (61−66) of this protein were thought to contribute to the stability of the interaction by binding within the minor groove.[65−68] This C-terminal arm bound within the minor groove and was able to approach the reactive C-1 hydrogens of the deoxyribose on either strand (Figure 15).[63] Therefore, OP was attached to the C-terminus by derivatizing Cro $A_{66}C$ with 5-(iodoacetamido)-1,10-phenanthroline.

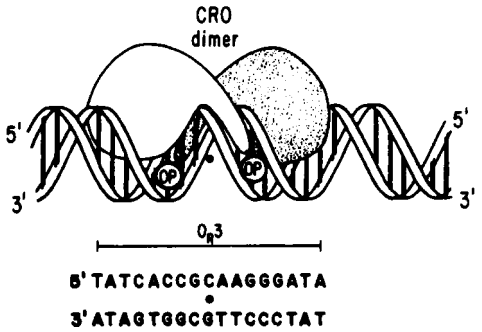


Figure 15. Schematic representation of complex between Cro A66C−OP and the opperator site OR-3. (Reprinted with permission from ref 63. Copyright 1991 American Chemical Society.)

Roughly 40% of the OR-3 site target site was cleaved within a 10 min incubation by the Cro $A_{66}C$−OP conjugate.[4] These results indicated that Cro $A_{66}C$−OP specifically cleaved the 17 bp OR-3 site. However, within the OR-3 site, this conjugate cleaved DNA in a virtually identical pattern to that of free OP−Cu. Two features of Cro $A_{66}C$−OP lead to this pattern. One was the high flexibility of the C-terminal arms, and the other was their antiparallel orientation within the dimeric protein. The studies of Cro $A_{66}C$−OP suggested that improvement of the OP-attached site-specific nucleases could be achieved by attaching the phenanthroline−copper to the DNA-binding protein using a less flexible tether and by positioning it to the 2-fold dyad axis of the target site.

On the basis of these lessons, a similar approach was used to prepare a trp repressor−OP conjugate.[69] The X-ray structure of the TrpR-*trp*EDCBA suggested that a derivative at residue 49 of *trp* repressor would direct the scission in a rigid fashion to the oxidatively sensitive minor groove at the dyad axis of the DNA-binding site.[57,58] An $E_{49}C$ mutant of TrpR was converted into a scission reagent by alkylation of the cysteine residue with 5-(iodoacetamido)-1,10-phenanthroline.[69] The $E_{49}C$ mutant placed OP−Cu at the N-terminal end of helix C. Therefore, the chemical nuclease could react with the minor groove without interfering with the DNA recognition mediated by the helix−turn−helix moiety of the *trp* repressor. It was found that TrpR $E_{49}C$−OP bound tightly to the *trp* repressor regulated operator sequences with a K_D of approximately 7×10^{-9} M. A

complex between TrpR $E_{49}C-OP$ and DNA was isolated by a gel-retardation assay using a labeled DNA fragment containing either the *aro*H or the *trp*EDCBA operator. Single-strand cleavage in the gel-shifted protein−DNA complexes with TrpR $E_{49}C-OP$ occurred with efficiencies of 80−90%.[70] Intriguingly, the rate for formation of double-stranded breaks was within a factor of 2−3 of that for single-stranded nicks.[69] Moreover, the yields for double-strand scission with this conjugate were close to 90% both in solution and in the gel matrix.[70]

The *E. coli* factor for inversion stimulation (Fis) protein was also converted into a DNA cleavage reagent. Although Fis is known as a site-specific DNA-binding protein, it lacks a well-defined consensus recognition sequence.[71] Previously, DNA footprinting data revealed a 15 base pair core sequence which was responsible for the Fis−DNA interaction. In addition, the electrophoretic mobility of Fis−DNA complexes and the Fis crystal structure indicated that significant DNA bending occurred upon Fis binding.[72,73] To evaluate the accuracy of this model and to determine the structural similarities between Fis complexes at different DNA recognition sites, Fis−OP conjugates $N_{98}C-OP$ and $N_{73}C-OP$ were prepared.[71] These conjugates were used for testing the cleavage pattern at four sites, the *hin* distal site, the proximal *hin* recombinational enhancer site, the *λ attR* site which functions in phage lambda excision, and the site I of the *rmB*P1 promoter. It was found that the relative scission locations cleaved by Fis-OP derivative at the four binding sites were virtually identical. This suggested that the overall mode of Fis interaction with target sites containing highly divergent DNA sequences was very similar. On the basis of DNA cleavage data obtained with $N_{98}C-OP$, a DNA bending angle between the sites cleaved by Fis $N_{98}C-OP$ was calculated to be 50°. A 70° bending angle observed with $N_{73}C$ indicated that the DNA within the flanking regions needed to be bent more than the original model suggested. Fis−OP derivatives thus provided a unique way for measuring DNA bendability that complements calculations derived from gel electrophoresis.

Additional studies with Fis-OP focused on identifying functional protein binding sites of biological interest within genomes.[74] The conjugates $N_{73}C-OP$ and $N_{73}C$-AOP containing a three-atom acetamido spacer and a seven-atom acetyl-*β*-alanylamino spacer, respectively, were synthesized. The derivative with the longer spacer was found to be less useful when applied to larger pieces of DNA such as the phage *λ* genome. This may have been due to the high background cleavage resulting from less specific binding by Fis. In contrast, $N_{73}C-OP$, which contained the shorter spacer, enabled the identification of a novel high-affinity Fis-binding site in the left operator of the *λ* regulator region. Another focus of the studies with Fis−OP was to improve the scission efficiency of such conjugates.[75] Insulation of the phenanthroline ring system from the amido group by methylene spacers enhanced DNA scission severalfold at the weaker cleavage sites. However, at strong cleavage sites, the methylene spacer did not increase cleavage efficiency. These results revealed that the efficiency of targeted scission was primarily a function of the orientation of the OP−Cu with respect to the oxidative C-1 hydrogen of the deoxyribose.

Ebright and co-workers used *E. coli* catabolite gene activator protein(CAP) as a scaffold to construct a DNA cleavage agent. CAP binds to a 22 base pair 2-fold symmetric DNA recognition site shown in Figure 16with extremely high affinity ($K_A = 4 \times 10^{10}$ M^{-1}). In their initial approach,[76] they covalently attached OP to amino acid 10 of the helix−turn−helix motif of CAP (Cys_{178}), which is the sole solvent-accessible cysteine residue of the CAP subunit.[77,78] *N*−Acetyl-5-amino-1,10-phenanthroline was covalently attached to Cys_{178} of the CAP using the procedure shown in Figure 17.[76]

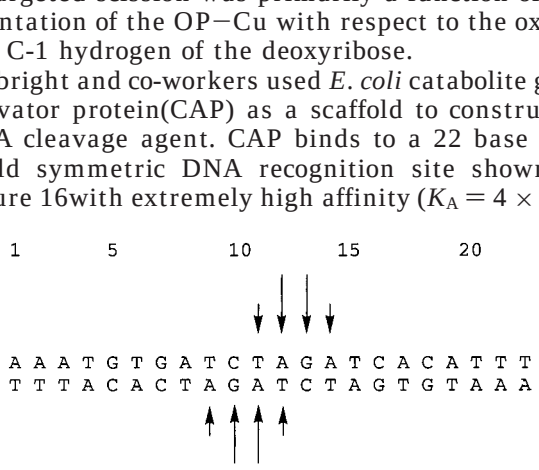


Figure 16. Site-specific DNA cleavage activity of [(*N*-acetyl-5-amino-1,10-phenanthroline)-Cys178]CAP. Arrows indicate the position at which DNA cleavage was observed.

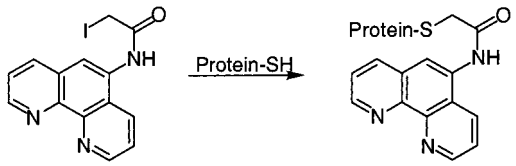


Figure 17. Reaction for the preparation of OP−Cu protein conjugates.

The product, CAP−OP178, exhibited a binding constant for the recognition site of 1×10^8 M^{-1}, thus indicating that the chemically modified CAP maintains substantial affinity for its cognate site, although this affinity is somewhat reduced relative to the unmodified protein. DNA cleavage was observed at four adjacent nucleotides which occupied 2-fold symmetry related positions within the 2-fold symmetric DNA recognition site (see Figure 16). No DNA cleavage was observed at other positions.[76] Moreover, CAP−OP178 was capable of yielding unique-site double-stranded cleavage of long DNA molecules (7164-bp).

In subsequent work, a different conjugate,[79] CAP−OP26, was constructed by first mutating the preexisting Cys_{178} to serine and introducing a new cysteine residue at position 26 of CAP. The cysteine residue was then alkylated by *N*-acetyl-5-amino-1,10-phenanthroline. Earlier work had shown that CAP bound to DNA and bent DNA in the specific CAP−DNA complex to an angle of approximately 90°, but it did not sharply bend DNA in the nonspecific CAP−DNA complex.[80,81] Thus, the conjugate, CAP−OP26 was able to distinguish the difference between DNA bending in the specific CAP−DNA complex and the nonspecific CAP−DNA complex. Additionally, analysis of scission reaction containing 7.2-kb and 48-kb DNA substrates showed that the cleavage was highly

specific. There was no detectable nonspecific cleavage of either the DNA substrate or the product DNA fragments. In addition, the scission reactions were highly efficient and proceeded to ≥90% completion. Even using megabase DNA (4.7-Mb), the reaction was still highly specific and occurred with efficiencies as high as 70%. These results revealed that CAP−OP26 had a high preference for specific cleavage compared with nonspecific DNA cleavage ($\geq 10^6$-fold preference) and had significant promise as a practical tool for chromosome mapping and chromosome cloning.[79]

Phenanthroline-containing conjugates have also been used to construct semisynthetic enzymes capable of hydrolytic chemistry. By attaching a phenanthroline ligand to Cys_{117}, present in the interior of ALBP (adipocyte lipid-binding protein), it was possible to position a Cu(II) atom within a chiral protein cavity (Figure 17). Fluorescence spectroscopy results and competition experiments indicated that ALBP−Phen bound copper with a greater affinity than free phenanthroline.[82] Interestingly, ALBP−Phen−Cu(II) was found to be able to promote the hydrolysis of several unactivated amino acid esters under mild conditions (pH 6.1, 25 °C) at rates 32- to 280-fold above the background rate in buffered aqueous solution; modest stereoselectivity was observed in these reactions. In 24-h incubations, 0.70−7.6 turnovers were obtained with enantiomeric excesses ranging from 31% to 86% ee. ALBP−Phen−Cu(II) was also able to catalyze the hydrolysis of picolinic acid methylnitroanilide (PMNA), an amide-containing substrate, at a higher reaction temperature (37 °C) and after longer incubation times. The rate enhancement for amide hydrolysis was found to be 1.6×10^4-fold higher than the background rate.[82] However, the value of k_{cat} obtained with ALBP−Phen−Cu(II) was 67-fold lower than that obtained with a Cu(II) bipyridine complex.[83] This rate decrease may reflect a nonoptimal (nonplanar) geometry for PMNA binding within the ALBP cavity due to steric interactions. The X-ray crystal structure of ALBP−Phen showed that protein could not accommodate the phenanthroline and PMNA within a planar conformation without significant distortion of the protein backbone.[84]

To examine how the site of 1,10-phenanthroline attachment influences hydrolytic selectivity, three residues at different locations within the protein cavity were chosen for the construction of new conjugates (Phen60, Phen72, and Phen104).[85] Using alanine isopropyl ester as a substrate, Phen60 catalyzed ester hydrolysis with less selectivity than ALBP−Phen while Phen72 was observed to promote the same reaction with higher selectivity. In contrast, hydrolysis of tyrosine methyl ester was catalyzed with higher selectivity by Phen60 and more rapidly by Phen104. These results indicated that the rate enhancement and substrate selectivity of hydrolysis reactions catalyzed by OP−Cu conjugates were largely dependent on the orientation and environment of the metal ligand within the protein cavity.

2. EDTA-Based Systems

Ferrous−EDTA is another metal chelate used for incorporating a transition metal into proteins. Interestingly, it was found that Fe−EDTA protein conjugates were not only able to cleave DNA or RNA, but were also capable of peptide bond hydrolysis.[86] The hydrolysis of protein backbones by Fe−EDTA protein conjugates does not depend on the chemical reactivity of the cleaved amino acid residues but appears to be sensitive to the proximity of the attached reagent to the protein. This characteristic is valuable and can be employed for sequence studies, functional analysis of structural domains, or for determining spatial relationships both within and between subunits in proteins where high-resolution structural information is not available.

On the basis of experimental observations,[87,88] one possible mechanism for peptide cleavage promoted by Fe−EDTA was proposed by Meares and Rana and is shown in Figure 18.[87]

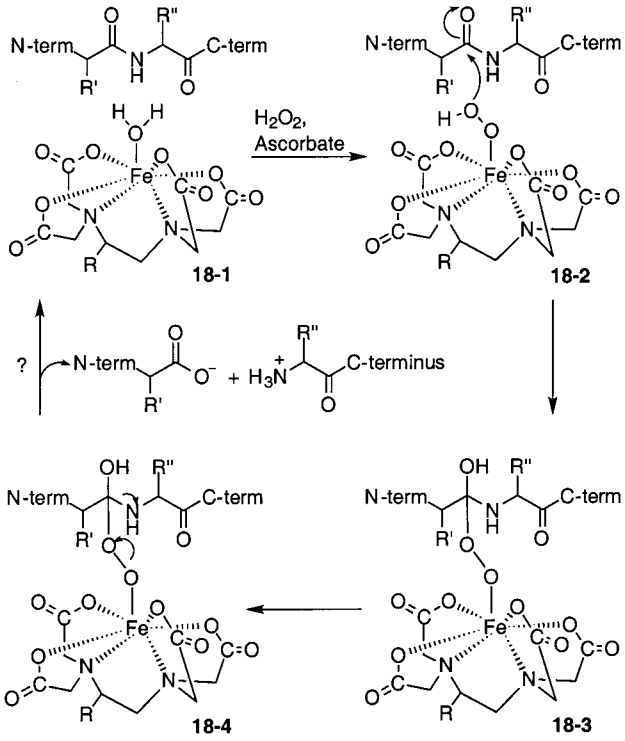


Figure 18. Possible mechanism for the observed proteolysis promoted by BABE conjugates.

The first step involves the direct binding of H_2O_2 to the Fe−EDTA and the formation of intermediate **18**−**2**. Alternatively, intermediate **18**−**2** may be formed by reduction of O_2 to H_2O_2 by ascorbate in the presence of the metal chelate.[89] Then, the peroxide on the intermediate **18**−**2** nucleophilically attacks the scissile amide bond and forms intermediate **18**−**3**. It should be noted that this attack occurs before the formation of a hydroxyl radical, which presumably arises from the decomposition of intermediate **18**−**2**. The final step is the C−N bond cleavage, concomitant with formation of a new carboxyl terminus formation. However, the step in which the iron complex is converted back to the original form is still unclear. Besides this hydrolytic cleavage mechanism, two oxidative mechanisms were also proposed.[90,91] The polypeptide backbone cleavage products resulting from these three different mechanisms are illustrated in Figure 19.

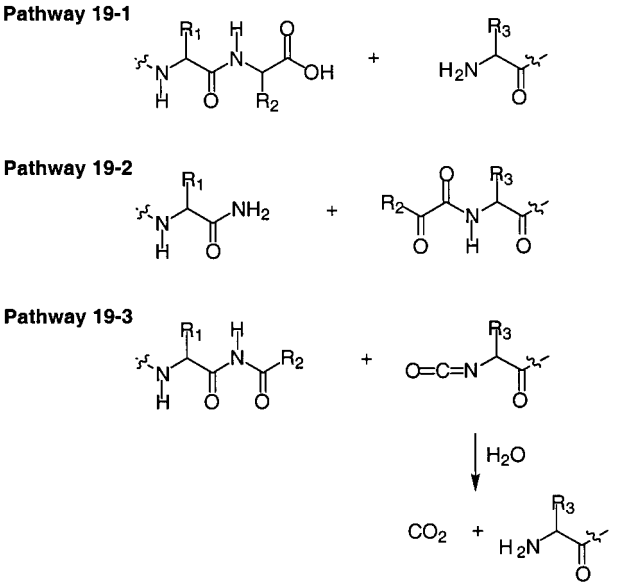


Figure 19. Putative reaction products resulting from protein cleavage by hydrolytic and oxidative pathways.

On the basis of electrospray mass spectrometric data, Ermácora and co-worker concluded that the peptide fragments were generated by at least two different cleavage mechanisms operating concurrently.

In the early stage of Meares' studies,[86−88,92,93] Fe−EDTA conjugates were constructed by alkylation of the free thiol of specific cysteine residues by (*S*)-1-[*p*-bromoacetamido)benzyl]−EDTA (BABE, **20**−**1**), shown in Figure 20, to form protein−BABE conjugates, followed by the addition of Fe^{2+} or Fe^{3+}.[94] The conjugates produced by this approach were able to perform intramolecular protein cleavage. Several different proteins including bovine serum albumin (BSA), human carbonic anhydrase I (HCAI), and cytochrome *bd* quinol oxidase of *E. coli* were used in these studies.[86−88,92] However, later research revealed that exogenous iron binds extensively to proteins, including RNA polymerase, at a variety of sites.[93] These additional metal-binding sites may act as contaminants and complicate the interpretation of cleavage experiments. To ensure that protein cutting was only due to the tethered Fe−EDTA, an improved reagent, Fe−BABE (**20**−**2**), was synthesized. Directly attaching Fe−BABE to the protein avoids the need to add iron after conjugation.

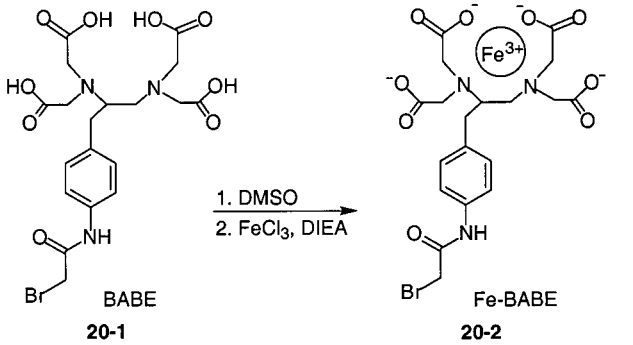


Figure 20. Structures of BABE and FeBABE.

E. coli RNA polymerase is composed of a four-protein core enzyme ($\alpha_2\beta\beta'$) and one of several possible σ subunits (the major sigma factor is σ^{70}). The core enzyme $\alpha_2\beta\beta'$ catalyzes RNA elongation, and the σ subunit provides the DNA promoter recognition activity. A cysteine residue was introduced into either a σ factor[95−99] or to the α subunit of *E. coli* RNA polymerase.[100] The resulting cysteine mutants were conjugated with Fe−BABE. These conjugates were then analyzed for their ability to cleave DNA or to hydrolyze the protein backbone. The results of these investigations indicated that Fe−BABE could be used for mapping the σ subunit contact sites on *E. coli* RNA polymerase and the promoter DNA sites proximal to conserved regions of σ factor.[95−100] These examples demonstrated that Fe−BABE protein conjugates are particularly well suited for probing protein structure.

Recently, another interesting approach to incorporate Fe−BABE to the surface of the σ^{70} protein has been investigated. A library of σ^{70} conjugates was generated by tethering Fe−BABE to surface-exposed nucleophilic residues, such as cysteine, lysine, or histidine, via 2-iminothiolane (2IT). Figure 21 illustrates the general scheme for creating and using this σ^{70} library.[101] The conjugates prepared from the resulting library contain an average of 1−2 cutting reagents per σ^{70} subunit. This library was used to

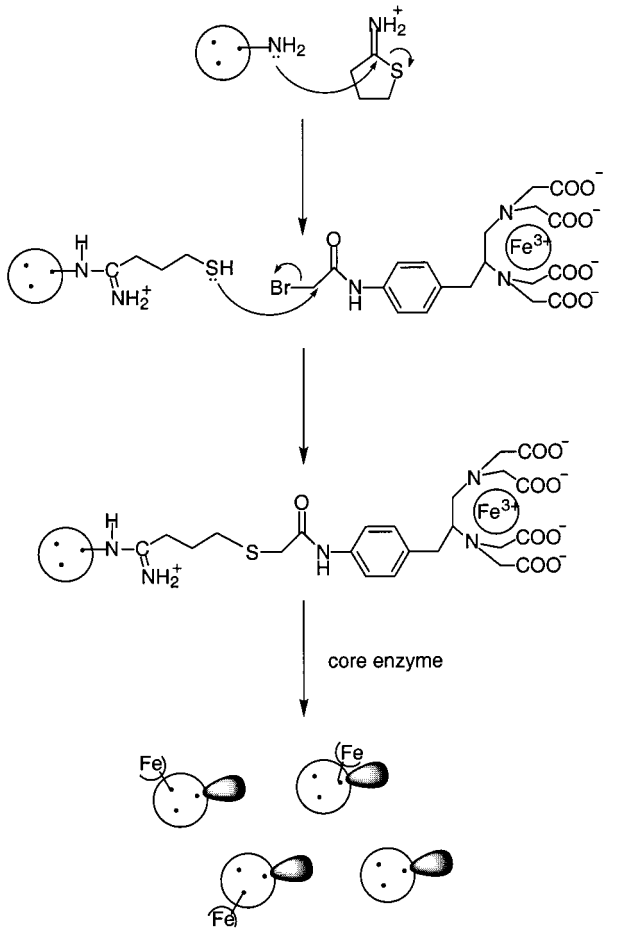


Figure 21. General scheme for creating and using a library of σ^{70} conjugates. Black dots represent surface-exposed reactive sites, and core enzymes ($\alpha_2\beta\beta'$) are shown as shaded ovals. σ^{70} was incubated with 2-fold excess of FeBABE over 2-iminothiolane in conjugation buffer. The reaction was incubated at 37 °C for 1 h.

identify the periphery of the σ^{70}-binding site on the core enzyme ($\alpha_2\beta\beta'$) of *E. coli* RNA polymerase. The experimental results obtained using this approach recapitulate and extend those obtained by single Fe−BABE protein conjugates. Thus, these experiments extend the utility of semisynthetic cleaving enzymes based on Fe−BABE.

An alternative approach for preparing protein−EDTA conjugates was developed by Ermácora and co-workers. In this case, an EDTA derivative containing an activated disulfide was prepared. The synthesis started with EDTA triethyl ester which was converted into cystamine−(EDTA triethyl ester)$_2$ upon carbodiimide-mediated coupling to cystamine. Hydrolysis of the ethyl esters, reduction of the disulfide, and activation with 2,2′-dipyridyl disulfide gave the desired compound (EPD, **22**−**1**) shown in Figure 22.

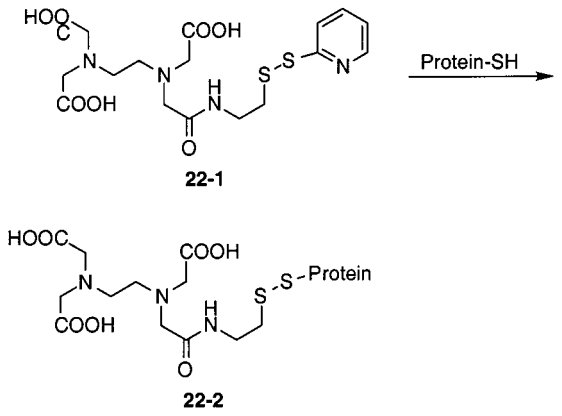


Figure 22. Structure of EPD and its reaction with protein cysteine residues.

The EPD reagent was used to prepare several EPD conjugates, **22**−**2**, that were investigated for their ability to undergo self-cleavage reactions.[102−104] These cases required ascorbate, covalently linked chelate, and oxygen.

EPD−Fe protein conjugates were used as DNA cleavage agents to provide valuable information for the study of protein−DNA interactions[104] The protein $\gamma\delta$ resolvase was covalently modified to form a $\gamma\delta$-EPD conjugate, and iron was subsequently added. As noted with other protein conjugates, DNA cleavage occurred only when the site of conjugation was proximal to the DNA and was limited to the immediate neighborhood of the EDTA-bearing residue. Results with these conjugates demonstrated that the DNA cleavage was highly localized and that the cleavage efficiency dropped off dramatically as a function of the distance between the EDTA−Fe complex and the DNA target.

For protein self-cleavage, EPD (**22**−**1**) was incubated with $FeCl_3$ to form an Fe−EPD complex prior to reaction with the protein. Then, the Fe−EPD protein conjugates were prepared by directly attaching the Fe-EPD complex to specific cysteine residues. A series of staphylococcal nuclease variants, each with a unique cysteine residue at distinct locations, was prepared.[102,103] Experiments with these constructs showed that the position of protein cleavage varied with the site of Fe−EPD attachment and depended on the conformation of the protein. Similar to the observations reported by Meares, protein self-cleavage was observed at residues in close proximity to the attachment site as well as the locations distant in sequence but brought close to the EDTA−Fe moiety by the protein fold. However, the cleavage yield was much lower (1−15%) and the number of cleavage products was greater with these EPD conjugates than with constructs incorporating BABE. These differences may be due to the fact that disulfide linkage is more sensitive to oxidative cleavage than the protein backbone.[90] Oxidative degradation of this linkage during the experiments may decrease protein cleavage. In addition, the greater hydrophilicity and conformational flexibility of EPD and the highly solvent-exposed site of attachment used with EPD may also be responsible for these differences.

Nevertheless, EPD was found to be small, flexible, and hydrophilic.[102] These properties limit the probability that the cutting reagent interacts with exposed hydrophobic regions of a protein and thus minimizes possible structural perturbations. The tethered EPD−Fe moieties only cleave the protein once. Moreover, the lifetime of the cleavage-promoting species should be much shorter than the time scale of protein conformational changes.[103] Thus, proteins incorporating EPD−Fe should be valuable probes of structure and conformation.

In an approach that employs chemistry distinct from those described above, Verdine and co-workers developed a method for conjugating EDTA site-specifically to proteins, regardless of protein size and amino acid composition.[105] The chemistry used in this method is a transesterification of an EDTA thioester derivative with an amino-terminal cysteine residue; the resulting product undergoes intramolecular rearrangement to produce an amide-linked EDTA as shown in Figure 23.

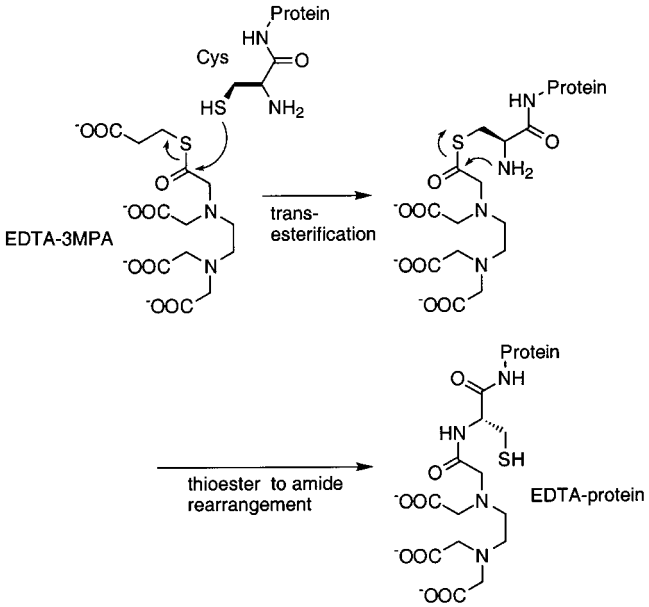


Figure 23. Conjugation of EDTA onto the amino-terminal cysteine residue of a protein through transesterification and subsequent acyl transfer. EDTA-3MPA represents EDTA-3-mercaptopropionic acid.

This acyl transfer reaction has been shown to be highly selective for an amino-terminal cysteine, even in the presence of internal cysteine and lysine residues. This selectivity derives from the irreversible rearrangement of the amino-terminal thioester linkage to the new amide. Although the EDTA thioester may react with the internal cysteine residue, these internal thioesters cannot further rearrange to the more stable amide. Eventually, they can be removed by a large excess of exogenous thiol present in the reaction mixture. The condensation between the EDTA thioester and the ϵ-amino group of internal lysine may also take place; however, at the pH used in acyl transfer reactions, these groups are present almost exclusively in the unreactive ammonium ion form. Furthermore, the aurthors found reactions with lysine residues could be supressed in the presence of high concentrations of dithiothreitol.

By reacting the thioester of EDTA with activator protein-1 (AP-1) peptides containing an amino-terminal cysteine residue, the resulting conjugates are affinity-cleaving reagents. They have been used to probe the interactions between AP-1 and the nuclear factor of activated T cells (NFAT).[105] AP-1 consists of two subunits, c-Fos and c-Jun, which heterodimerize and bind DNA. Each subunit contains two functional segments: an amino-terminal basic region which directly contacts DNA and a carboxy-terminal leucine zipper responsible for dimerization. On the basis of electrophoretic mobility shift assays, it was found that the EDTA-conjugated proteins bound DNA in a similar manner as the unmodified proteins. The results of affinity cleavage experiments reveal that the leucine zipper is solely responsible for determining the specificity of the protein−protein interactions between NFAT and AP-1.

This conjugation method, based on acyl transfer chemistry, permits the generation of affinity-cleaving reagents without the need to use synthetic peptides and proteins or the requirement that the protein has a single cysteine residue. It should be noted that many proteins contain zinc-bound cysteine residues that cannot be mutated without loss of function; the present method could allow these proteins to be analyzed using the affinity-cleaving method. Furthermore, not only EDTA can be conjugated onto proteins via the acyl transfer reaciton; other functional groups such as fluorescein, biotin, or other catalytic moieties may also be attached to proteins in a similar manner.

3. A Protein Incorporating a Salen Complex

As noted above, protein conjugates containing metal ions have been prepared for performing asymmetric catalysis. Recently Lu and co-workers developed a system for epoxidation of unfunctionalized olefins. This was accomplished by incorporating a Mn(III)(Salen) complex, an achiral version of the well-known Jacobsen's catalyst, into cytochrome *c* peroxidase (CcP). The stereoselectivity of the resulting semisynthestic enzyme, Mn(III)Salen−CcP, showed approximately 50% ee with styrene as the substrate. Using *cis*-β-methylstyrene and *cis*-2-heptene as substrates, extensive turnovers of 400 and 200, respectively, were obtained.[106,107]

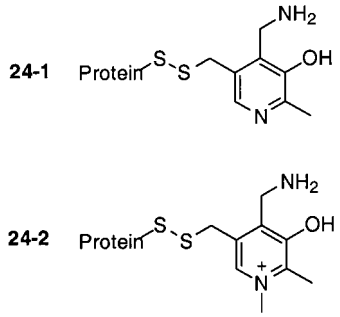


Figure 24. Schematic representation of protein−pyridoxamine conjugate 24−1 and protein−MPx conjugate **24**−**2**.

B. Nonmetal-Containing Constructs

1. Transaminases

Distefano and co-workers adopted a host−guest approach to developing artificial enzymes that involves the introduction of catalytic functionality into a protein cavity-derived host. In particular, they employed a family of small lipid-binding proteins for their semisynthetic enzyme design. These proteins are members of a class of polypetides whose structures consist of two orthogonal planes of β-sheet and an α-helical lid. A variety of fatty acids ranging from palmitate to arachidonate bind in a large (600 Å^3) cavity formed between the two sheets. Structural characterization, including X-ray crystallographic data and NMR measurement, provides a clear picture of the protein structures.[108,109] Initially, Kuang and co-workers[110] chemically attached a pyridoxamine analogue to adipocyte lipid-binding protein (ALBP) at Cys_{117} through a disulfide linkage, resulting in the formation of a construct denoted ALBP−PX (Figure 24, **24**−**1**).

This semisynthetic biocatalyst reductively aminated a variety of α-keto acids to amino acids shown in Figure 25, with enantioselectivities ranging from

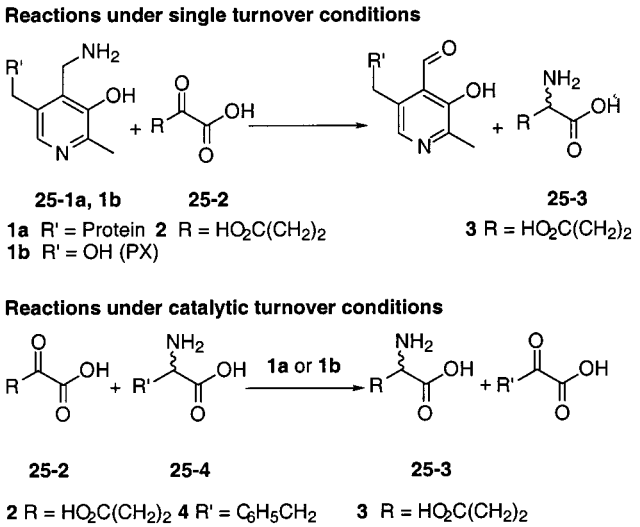


Figure 25. Transamination reaction catalyzed by protein−PX conjugate under single turnover and catalytic conditions.

0% to 94% ee under single turnover conditions. The reaction rates of these reactions were not, however, significantly faster than those involving free pyri-

doxamine (**25**−**1b**). This suggested that the protein cavity was functioning as a chiral environment that controls the facial selectivity of the protonation of the aldimine intermediate without forming specific interactions with the bound pyridoxamine cofactor to accelerate the reaction, as confirmed by an X-ray crystal structure.[84] Modeling of the Schiff base complexes with several amino acids indicated that one face of the putative aldimine intermediate was protected against the approach of the solvent or buffer molecules that must be the proton source for the reaction, given the lack of suitable functional groups within the cavity. This structural data provided a rationale for explaining the enantioselectivity observed in the ALBP−PX system.

To examine the effect of altering the cofactor-attachment site, a different fatty acid-binding protein, intestinal fatty acid-binding protein (IFABP), was utilized. The wild-type IFABP has no Cys inside the cavity, and therefore, it is an ideal template to introduce single Cys residues at different positions.[111] Cys mutations at various positions ($V_{60}C$, $L_{72}C$, and $A_{104}C$) were introduced using site-directed mutagensis as illustrated in Figure 26.

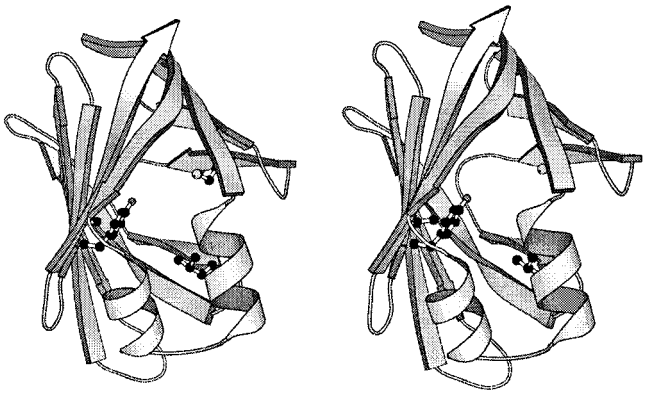


Figure 26. Stereoview from the X-ray structure of IFABP showing the side chain locations of residues V_{60}, L_{72}, Y_{117}, and A_{104} in ball-and-stick representations (listed clockwise from upper right).

Three conjugates (IFABP−PX60, IFABP−PX72 and IFABP−PX104) were prepared and studied for transamination ability under single turnover conditions. Interestingly, each mutant conjugate exhibited a unique pattern of reactivity and selectivity. Compared to ALBP−PX, IFABP−PX60 reacted at least 9.4-fold more rapidly, while IFABP−PX72 inverted the enantioselectivity of reactions, and IFABP−PX104 displayed selectivity for unbranched substrates.[112] Therefore, by varying the position of the cofactor attachment and thereby changing the microenvironment of the "active site", the rate, enantioselectivity, and substrate selectivity could be modulated effectively by employing site-directed mutagenesis in conjunction with covalent modification.

IFABP−PX60 was subsequently studied in further detail because of its increased transamination rate compared to ALBP−PX and free pyridoxamine systems.[113] Under single turnover conditions, IFABP−PX60 (**25**−**1a**) converted α-keto glutarate (**25**−**2**) to glutamic acid (**25**−**3**) 62-fold faster than free pyridoxamine (**25**−**1b**), clearly indicating that the protein host was playing an important role in the reaction. Under catalytic conditions, using tyrosine or phenylalanine as the amino source to recycle the cofactor from the aldehyde form back to amine form, L-glutamate was formed with an enantiomeric purity of 93% ee. As many as 50 turnovers were observed with long reaction times (14 days). Analysis of the k_{cat} and K_M values for these reactions summarized in Table 1 indicated a 200-fold increase in the catalytic reaction efficiency compared to free pyridoxamine. It also suggested that the accelerated catalysis observed occurred primarily due to an increase in substrate binding (50-fold) together with a smaller effect on the maximal rate (4-fold). The overall magnitude of k_{cat}/K_M (0.16 h^{-1} M^{-1}) indicates that this catalyst is still quite primitive compared to natural enzyme systems, although these results clearly show progress in the right direction.

Table 1. Kinetic Data for IFABP−PX and MPX Conjugates Using α-Keto Glutarate and Phe as the Substrates

conjugate	K_m (mM)	K_{cat} (h^{-1})	K_{cat}/K_m (h^{-1} mM^{-1})
PX	73	0.032	4.4×10^{-4}
MPX	38.7	0.031	8.0×10^{-4}
IFABP−PX60	1.8	0.29	0.16
IFABP-MPX60	6.8	0.23	0.034
hsIFABP−PX60	10.2	0.22	0.022
IFABP−PxK38	0.81	0.44	0.54
IFABP-MPxK38	13.7	1.12	0.08
IFABP−PxK51	0.24	0.44	1.83
IFABP-MPxK51	8.9	0.52	0.06
IFABP−Px126	5.5	0.18	0.03
IFABP-MPx126	40	1.10	0.03
IFABP−Px126/14	6.0	0.11	0.02
IFABP-MPx126/14	48	0.78	0.02

One means of increasing the rate of pyridoxamine-catalyzed reactions is to include metal ions in the reaction system. Metal ions are believed to stabilize the formation of the Schiff base intermediates and increase the acidity of the protons that must be removed in the reaction. The addition of metal ions to reactions catalyzed by ALBP− and IFABP−pyridoxamine conjugates resulted in both positive and negative perturbations of the reaction rate.[114] IFABP−PX104 reacted 4.7-fold faster in the presence of copper(II), whereas IFABP-PX60 reacted 4.4-fold slower; both of these rate effects were accompanied by a decrease in reaction enantioselectivity. Little change was observed for the reaction catalyzed by IFABP−PX72. UV−vis spectroscopy experiments suggested that IFABP−PX60 and IFABP−PX104 but not IFABP−PX72 formed a complex with Cu(II). Thus, metal ions can be used to increase semisynthetic enzyme efficiently, although this did not occur in all cases.

Introducing a permanent positive charge to the N atom in the pyridoxamine ring was also shown to influence the reaction rate in different directions. In some model systems, an *N*-methylated species was shown to accelerate the transamination rate up to 20-fold. In contrast, after reconstitution with *N*-methyl pyridoxamine, alanine aminotransferase lost its activity almost completely (greater than 99.8%). Therefore, N-quarternization may have both a positive and negative influence on the reaction rate.

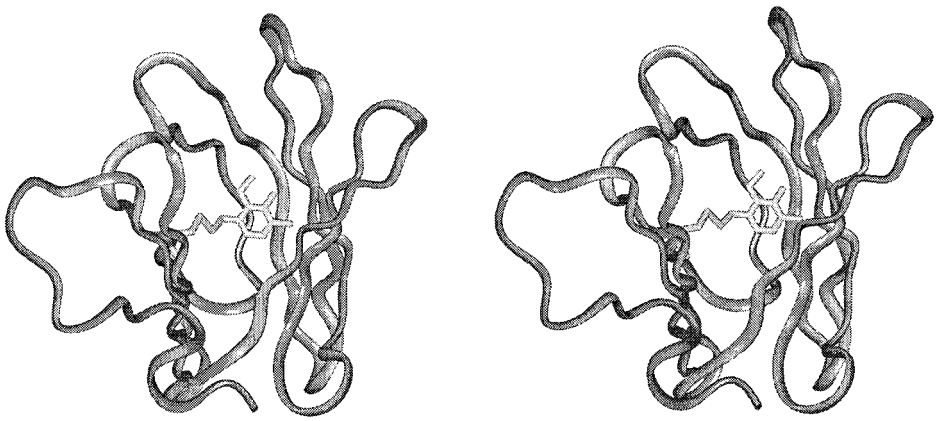


Figure 27. Stereoview of a model of the hsIFABP−PX conjugate based on the structure of hsIFABP determined by NMR.

IFABP-MPX60 (structure **24-2**) was prepared[115] and under catalytic conditions, 5.8 turnovers were observed using α-keto glutarate and phenylalanine as the substrates. Under these conditions, an enantioselectivity of 41% ee was obtained. Kinetic analysis of the reaction indicated that in comparison to IFABP−PX60, IFABP-MPX60 showed higher values (1.1- to 3.8-fold) for K_M' whereas the k_{cat} values were similar for both catalysts. This suggested that N-methylation had no positive effects on the reaction rate, which also suggested that one or more of the deprotonation steps were not rate limiting in the IFABP−PX-catalyzed reactions. However, given that proton transfer events are frequently rate determining in natural transaminases, it is likely that MPX reagent will be useful in future catalyst design.

Another possible step in catalysis promoted by IFABP-based semisynthetic enzymes that maybe rate determining is the entry or exit of substrates and/or products from the protein cavity. A helixless (hs) IFABP mutant was prepared by deleting 17 helical residues (15−31) from the N-terminus and replacing them with a dipeptide linker (Ser-Gly) using site-directed mutagenesis.[116] NMR study previously showed that this mutant preserved the original β-sheet secondary structure without the α-helical lid and was still relatively stable.[117] After attaching a pyridoxamine cofactor or its *N*-methylated counterpart to a cysteine residue in the cavity of hsIFABPV60C, modeled in Figure 27, catalysis was investigated under catalytic turnover conditions.[118] In 24 h, approximately two turnovers were observed in reactions containing hsIFABP−PX60; in those reactions, a selectivity of 93% ee was observed, comparable to results seen with IFABP−PX60. Kinetics studies indicated that k_{cat} values were comparable for both IFABP−PX60 (0.20 h^{-1}) and hsIFABP−PX60 (0.22 h^{-1}), while hsIFABP−PX60 exhibited a 4-fold increase in K_M. After introducing the MPX cofactor into the cavity of the helixless protein, 4.9 turnovers in 24 h with 35% ee were observed for the production of Glu (**25**−**3**) under the same conditions. Taken together, these results suggested that the removal of the α-helical lid did not affect the maximal rate and enantioselectivity of the respective constructs. However, deletion of this structural element did decrease the substrate-binding affinity as evidenced by an increase in the K_M value. Thus, although the removal of the helical lid did not offer significant advantage in the pyridoxamine system, it may be useful as a template to introduce larger coenzyme factors or for performing reactions in nonaqeuous solvents.

To further imitate natural aminotranferases, lysine residues were introduced into the protein cavity to allow Schiff base formation with the pyridoxal in the reaction cycle as shown in Figure 28.

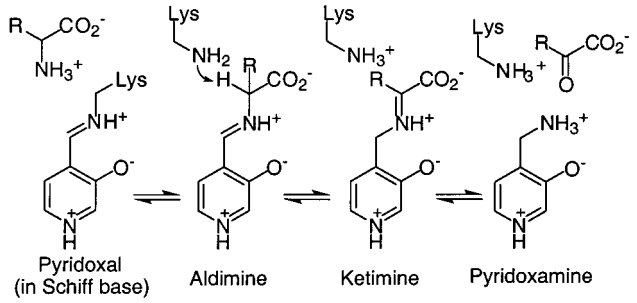


Figure 28. Simplified mechanism for the participation of a lysine residue in a transamination half reaction.

These residues can also act as a general base and a general acid (in the protonated form) in the transaminantion process. On the basis of the crystal structure of IFABP, molecular modeling was performed to identify optimum positions to introduce such a functional group. Mutants $L_{38}K/V_{60}C$ and $E_{51}K/V_{60}C$ were identified and therefore generated using site-directed mutagenesis.[119] The resulting assemblies IFABP−PxK38 and IFABP−PxK51 (Figure 29) showed improved values for k_{cat} and K_M. The overall catalytic efficiency (k_{cat}/K_M) of IFABP−PxK51 increased 4200-fold compared to the unliganded pyridoxamine phosphate (**25**−**1b**) and was 12-fold greater compared to IFABP−PX60 while maintaining comparable enantioselectivity (83−94% ee). The predominant kinetic effect of the lysine residues is on K_M. Both conjugates manifested a moderate decrease in K_M values (2.2- and 7.5-fold), while a small increase in k_{cat} (1.5-fold) was observed. UV−vis spectroscopy, fluorescence, and electrospray mass spectrometry verified the catalytic function (the formation of the Schiff base) of the introduced lysine residue in the reaction process. The only modest rate increase resulting from introduction of lysine is likely due to the absence of additional residues normally

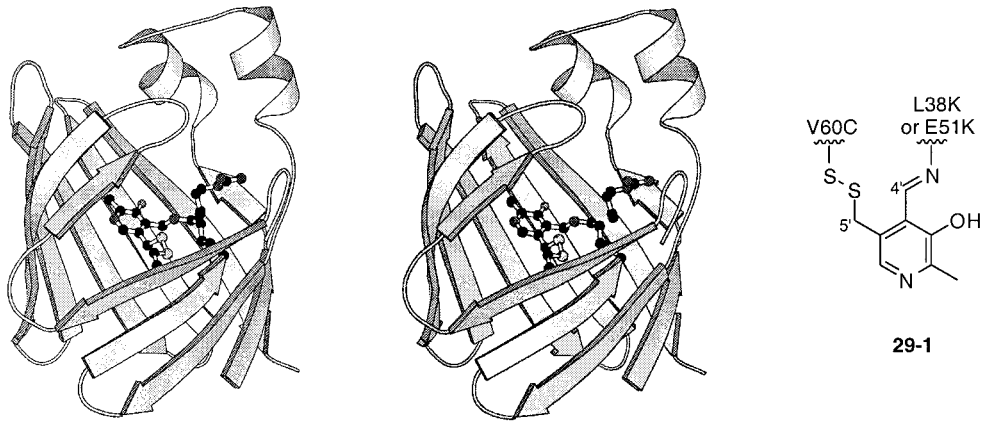


Figure 29. Stereoview of a computational model of IFABP−PxK51 developed from the crystal structure of IFABP. The pyridoxal is bound via a disulfide bond to Cys_{60} and a Schiff base linkage to Lys_{51}; a cationic residue (Arg_{126}) is close to the catalytic center.

present in transaminases that modulate cofactor/substrate reactivity. Nevertheless, these studies clearly demonstrated that features of enzymatic processes including covalent catalysis could be mimicked successfully.

To improve the activity of the above two Lys mutants, glutamic acid was introduced within proximity to the pyridoxamine pyridine nitrogen using molecular modeling and site directed mutagenesis.[120] Such an arrangement is observed in natural transaminases and is thought to enforce pyridine protonation. However, the resulting two mutants (IFABP−$V_{60}C/L_{38}K/F_{93}E$ and −$V_{60}C/E_{51}K/F_{93}E$) were unstable. Therefore, N-methylated pyridoxamine was incorporated to IFABP−$V_{60}C/L_{38}K$ and −$V_{60}C/E_{51}K$ to mimic this effect. The resulting conjugates, IFABP-MPxK38 and IFABP-MPxK51 (**24**−**2**), were able to catalyze the transamination reaction between α-ketoglutarate and various amino acids. Within 24 h of the reaction time up to 17 catalytic turnovers were achieved yielding L-glutamate with up to 96% ee. Kinetics studies indicated that the k_{cat} values (1.12 h^{-1} for IFABP-MPxK38 and 0.52 h^{-1} for IFABP-MPxK51) and turnover numbers (12.2 turnovers by IFABP-MPxK38 and 5.7 turnovers by IFABP-MPx51 in 24 h) observed with these constructs under standard conditions are the highest achieved to date in this system. However, their K_M values were up to 37-fold higher than those for the nonmethylated IFABP−PxK38 and −PxK51. Molecular modeling suggests that the N-methyl group attached to the pyridoxamine was too close to the phenyl ring of Phe_{93}, forcing the cofactor to shift position which in turn perturbed important interactions between the protein and the substrate. The limited space within the cavity and especially its substrate−ketimine form might be the reason for the decreased binding affinity. These modeling results provided a good explanation for the observed decrease in substrate-binding affinity.

Computational methods were also utilized to explore the interactions between the active site residues from IFABP−PX60 and reaction intermediates using α-keto glutarate as the substrate. Arg_{126} and Tyr_{14} were identified as two important residues which interacted with the γ-carboxylate group of α-keto gluatarate from the modeling results shown in Figure 30.

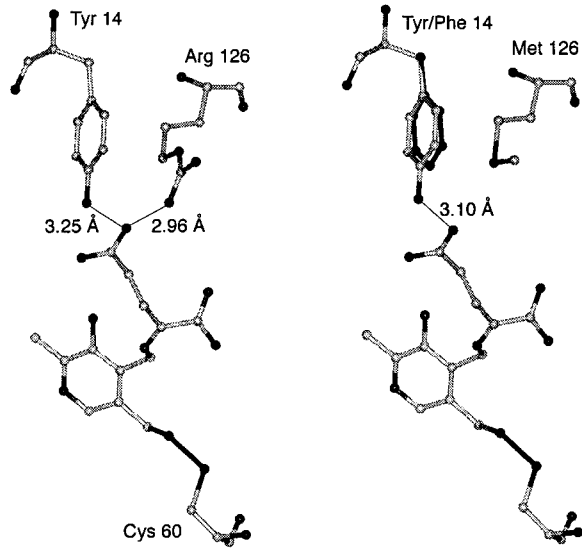
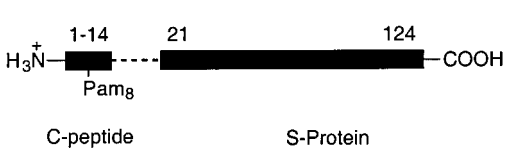


Figure 30. Computer models of the IFABP−P active site with α-ketoglutarate bound in the ketimine form. The γ-carboxylate group of the substrate interacts with R_{126} and Y_{14} (left). The mutants $R_{126}M$ or $R_{126}M/Y_{14}F$ (black), respectively, are shown on the right side for comparison.

Mutants IFABP−$V_{60}C/R_{126}M$ and −$V_{60}C/R_{126}M/Y_{14}F$ were prepared, and pyridoxamine was attached to each mutant protein.[121] Interestingly, IFABP−PxM126 had a K_M 3-fold higher than IFABP−PX, while the second mutation at position 14 had no significant effect. The k_{cat} values for both conjugates decreased by 40% compared to the original IFABP−PX60. N-Methylated pyridoxamine conjugates were also introduced into these two mutants. Compared to the original IFABP-MPX60 construct, the mutations in positions 126 and 14 increased the K_M values up to 7-fold. Interestingly, IFABP-MPxM126 had a k_{cat} value about 5-fold higher. In general, the above work illustrated how a combination of molecular modeling, site-directed mutagensis, protein chemistry, and kinetic analysis could be used to design new catalytic systems.

As noted above, RNase S can be reconstituted with modified peptides to yield RNase with altered properties. This can be performed with S-peptide (residues 1−20) or with a shorter version denoted as C-peptide (residues 1−14) as shown in Figure 31.

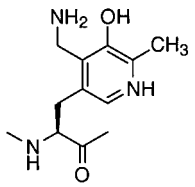
31-1



31-2

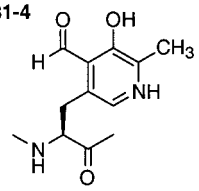
a CP: Lys-E-T-A-A-A-Lys-Phe-E-R-Q-H-Met-D-NH_2
b H1: Nle-E-T-A-A-A-Gly-Pam-E-R-Q-H-Nle-D-NH_2
c S1: Nle-E-T-A-A-A-Ala-Pam-E-R-Q-S-Nle-D-NH_2
d C1: Nle-E-T-A-A-A-Gly-Pal-E-R-Q-H-Nle-D-NH_2
e C2: Nle-E-T-A-A-A-Gly-Pal-E-R-Q-H-Nle-D-NH_2

31-3



Pam

31-4



Pal

Figure 31. Schematic representation of Pam- or Pal-incorporated into a C-peptide and S-protein complex. Peptides H1 and S1 contain Pam at position 8, while C1 and C2 have Pal at position 8.

RNase S-protein can also be reconstituted with peptides bearing other catalytic functionality to impart new enzymatic functions. On the basis of the C-peptide sequence, Imperiali and Roy[122] first introduced a pyridoxal cofactor (Pal) into position 8 in the C-peptide fragments as shown in structure **30-1** to be in the proximty to the general acid−base pair, His_{12} and His_{119}. The idea was to use the S-protein to modulate the pyridoxamine environment. The lysine residues in native C-peptide were replaced with glycine **31**−**2b** (peptide C1, Figure 31) or norleucine **3**−**2c** (peptide C2, Figure 31) to prevent the intramolecular Schiff base formation with the coenzyme-derived aldehyde. Both peptides bound with S-protein shown in Figure 32 with affinities compa-

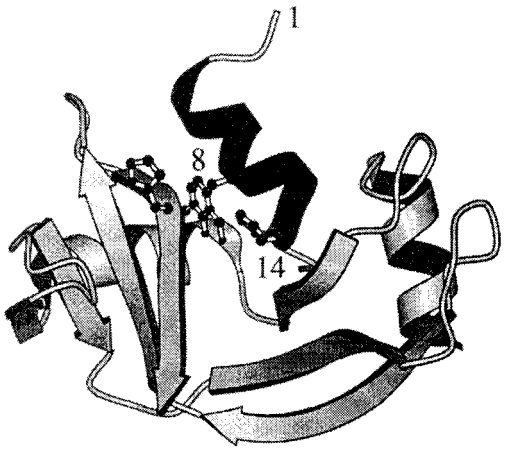


Figure 32. RNase-S complex showing the proximity of Pal/Pam residues to His residues in RNase S-protein. (Reprinted with permission from ref 122. Copyright 1994 American Chemical Society.)

rable to that of native C-peptide. These protein complexes were then evaluated for their ability to convert L-alanine to pyruvate under single turnover conditions in the presence or absence of copper(II). Interestingly, in one experiment, k_{obsd} for C1−SP was 16.7-fold higher than that for the C1 peptide alone. Replacement of a single residue (Gly_7 to Nle_7) resulted a 12-fold drop in the transaminase activity relative to the C1−SP complex, suggesting that relatively bulky Nle residue might disrupt the complex between the peptide and protein. This also suggested that precise positioning of the pyridoxal unit was important for efficient reaction.

In a similar approach, pyridoxamine was introduced into a C-peptide at position 8 to maintain the interactions with His_{12} and His_{119}.[123] Again, the peptide-derived Lys residue was substituted with Gly for the same reason as in the design for the Pal-peptide, resulting in a peptide denoted H1 **31**−**2d** (Figure 31). Upon the formation of RNase complex H1−SP, a 7-fold rate enhancement was observed compared to uncomplexed peptides under single turnover conditions. However, after replacing the His residue at position 12 with Ser **31**−**2e** (Figure 31), only a 3-fold rate increase was observed for S1−SP. Under catalytic conditions with pyruvate and L-phenylalanine as the substrates, uncomplexed peptides did not show catalytic turnover, suggesting that a hydrophobic microenvironment in the peptide−protein complex is critical for the catalysis. However, in the presence of the S-protein, catalysis ensued. Up to 1.5 turnovers were observed in 160 h from S1−SP complex. Significantly, the enantioselectivity of the alanine product from S1−SP complex was 31% ee (D-alanine) compared to 15% *ee* (L-alanine) with H1−SP complex. This suggests that His_{12} is very important in the chiral induction. Although these RNase-based systems are still primitive, they clearly demonstrate that peptide synthesis can be exploited to selectively position catalytically important residues within a protein complex.

2. Flavoenzymes

Flavin analogues were used as modification agents for the preparation of semisynthetic enzymes because of their known catalytic versatility including hydride transfer, hydroxylation, and monooxygenation.[124] Even model flavins can be quite effective catalysts, so it seemed that flavoenzymes could be obtained by chemical modification of an existing protein without necessitating involvement of specific amino acid functional groups in the enzyme for the catalysis. Early work on semisynthetic flavin enzymes focused on papain as the protein scaffold. Papain, based on its X-ray structure,[125−127] was known to possess an active site groove that was about 25 Å long. Furthermore, it was well established that this enzyme has a uniquely reactive thiol group (Cys_{25}) in its active site that can be selectively alkylated by a variety of reagents.[128−130]

Semisynthetic flavopapin was initially demonstrated to be able to catalyze the oxidation of dihydronicotinamides.[131−136] The first two semisynthetic flavoenzymes, flavopapain **33**−**1a** and **33**−**2a,** were generated in the late 1970s (Figure 33).[131]

On the basis of model building, flavopapain **33**−**1a** has a bulky ribityl group at N-10 and this ribityl

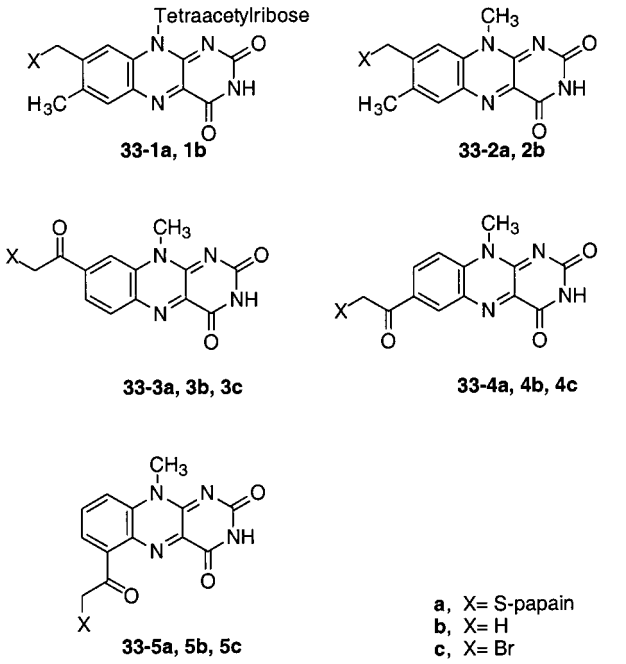


Figure 33. Flavin analogues used for the preparation of flavopapains.

group has deleterious interactions with the interior of the papain molecule. Even when the ribityl group was replaced by a much smaller methyl group at N-10 to form flavopapain **33**−**2a**, this isoalloxane derivative was still too large to remain fixed within the binding site. This may be due to excessive flexibility of the flavin ring in the active site. Kinetic studies indicated that **33**−**1a** and **33**−**2a** did not exhibit saturation kinetics in the oxidation of *N*-benzyl-1,4-dihydronicotinamide and *N*-propyl-1,4-dihydronicotinamide at substrate concentrations as high as 10 mM. Also, the second-order rate constants for the oxidation of the 1,4-dihydronicotinamide by **33**−**1a** and **33**−**2a** show only 3-fold rate accelerations compared to the free flavin analogues **33**−**1b** and **33**−**2b**.

To constrain the mobility of the flavin ring attached to the Cys_{25}, additional hydrogen-bonding interactions were incorporated into the design. Flavopapains **33**−**3a**, **33**−**4a**, and **33**−**5a** shown in Figure 32 were obtained by alkylation of the active site of papain by the corresponding brominated derivatives **33**−**3c**, **33**−**4c**, and **33**−**5c**.[132,133,135,136] The carbonyl oxygen of the acetyl substituent attached to the flavin ring system was designed to be in close proximity to two potential hydrogen-bond donating groups, the backbone NH of Cys_{25} and a side chain NH of Gln_{19}. Such hydrogen-bond interactions could aid in constraining the covalently bound flavin to the interior of the enzyme close to the substrate-binding site.

Tables 2 and 3 present the kinetic parameters for the oxidation of nicotinamides catalyzed by flavopapains **33**−**3a**, **33**−**4a**, and the corresponding free flavin analogues under aerobic conditions.[132,136] From Table 2 it can be seen that the most effective flavopapain, **33**−**3a,** accelerates the oxidation of *N*-benzyl-1, 4-dihydronicotinamide and the corresponding *N*-propyl compound by 200-fold and 67-fold compared to the second-order rate constants for the corresponding oxidation reactions catalyzed by the model flavin **33**−**3b**. Furthermore, flavopapain **33**−**3a** achieved a 670-fold rate acceleration for the oxidation of *N*-hexyl-1,4-dihydronicotinamide. The kinetic parameter k_{cat}/K_M for this substrate oxidation is 570 000 M^{-1} s^{-1}, which is even larger than the corresponding value for several naturally occurring flavoenzymes, such as NADH-specific FMN oxidoreductase, old yellow enzyme, and glucose oxidase.[137−139] Flavopapain **33**−**4a** was a moderately effective flavoenzyme (Table 3).

Table 2. Kinetic Parameters for the Oxidation of Dihydronicotinamides by Flavopapain 32−3a and Flavin Analog 32−3b

	parameters for reactions			
	enzymatic (**32**−**3a**)			model (**32**−**3b**)
substrate	K_m (M)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	k_2 (M^{-1} s^{-1})
NBzNH	2.7×10^{-6}	0.093	33 800	170
NPrNH	0.81×10^{-6}	0.048	58 700	878
NHxNH	0.12×10^{-6}	0.067	570 000	917
NADH	340×10^{-6}	0.0073	21	5

Table 3. Kinetic Parameters for the Oxidation of Dihydronicotinamides by Flavopapain 32−4a and Flavin Analog 32−4b

	parameters for reactions			
	enzymatic (**32**−**4a**)			model (**32**−**4b**)
substrate	K_m (M)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	k_2 (M^{-1} s^{-1})
NBzNH	1.9×10^{-4}	0.64	3 370	185
NEtNH	1.3×10^{-4}	0.72	5 500	853
NPrNH	1.0×10^{-4}	0.81	8 100	845
NHxNH	0.42×10^{-4}	0.44	10 500	843

Comparing the kinetic parameters of flavopapain **33**−**4a** with the second-order rate constants for the model flavin shows that there was an approximately 20-fold rate acceleration for the enzymatic system. In contrast to flavopapain **33**−**3a** and **33**−**4a**, flavopapain **33**−**5a** was an extremely poor catalyst for the oxidation of dihydronicotinamide. The k_{cat}/K_M value for the oxidation of *N*-benzyl-1,4-dihydronicotinamide was 41 M^{-1} s^{-1}, which was even less than the second-order rate constant (64 M^{-1} s^{-1}) of the corresponding model reaction. These results indicate a critical role for proper placement of the isoalloxazine moiety in the active site.

Figure 34 shows a proposed mechanistic pathway for the oxidation of dihydronicotinamides by flavopapains.[1] Structure **33-1** illustrates the resting state of the flavoenzyme. The flavin participates in a charge-transfer complex with the indole side chain of Trp_{26}. The carbonyl group of the acetyl side chain on the flavin ring system is hydrogen bonded to the backbone NH of Cys_{25} and a side chain of Gln_{19}. Structure **33-2** shows a *N*-alkyl-1,4-dihydronicotinamide which is bound within the hydrophobic cavity of the papain and corresponds to the formation of a Michaelis complex. When the ES′ intermediate is generated (structure **33-3**), the charge-transfer complex existing between the flavin moiety and the indole ring in Trp_{26} is disrupted and the flavin moiety moves to a distinctly different environment. After the realignment of the flavin moiety has taken place

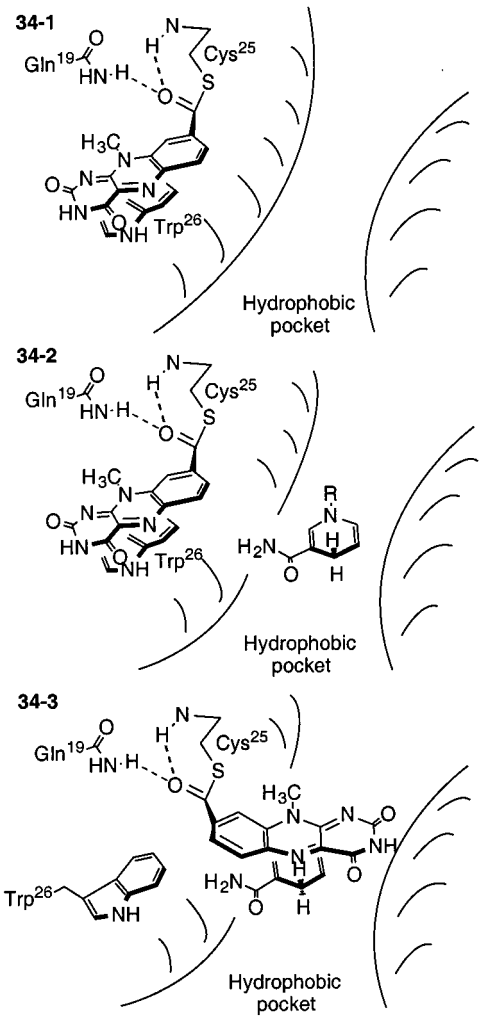


Figure 34. Mechanistic pathway for the oxidation of dihydronicotinamides by flavopapains.

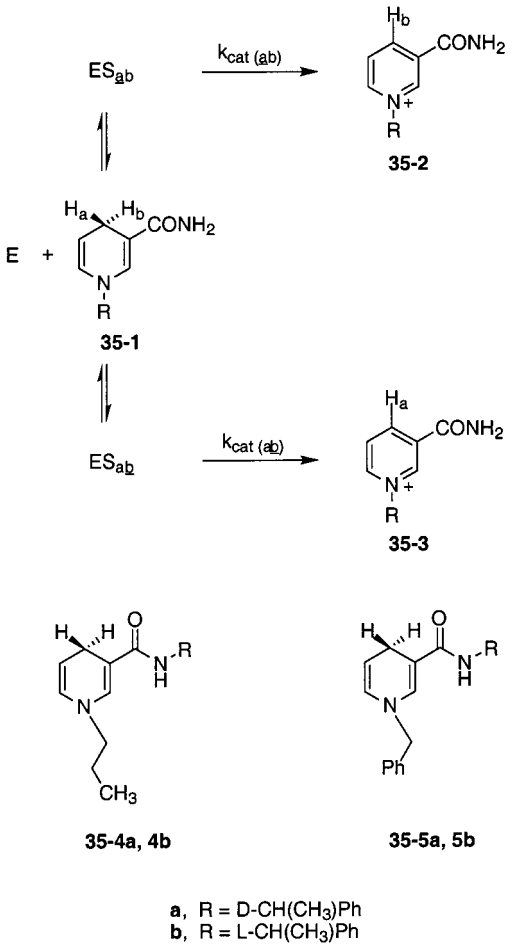


Figure 35. Kinetic scheme for hydride transfer from dihydronicotinamide and chiral dihydronicotinamide derivatives.

giving ES′, the redox reaction, in which hydride transfer occurs from the dihydronicotinamide to the flavin, can take place.

The formation of the ES′ intermediate explains the relative kinetic behavior of **33**−**3a**, **33**−**4a**, and **33**−**5a**. Flavopapains undergo the rotation step leading to ES′. The N-5 atom of the flavin ring is in a highly favorable alignment to receive a hydride from the substrate. However, when the flavin is attached to the papain through position 7 of the ring system (**33**−**4a**), this orients the N-5 atom in a less optimal manner than in **33**−**3a**. In contrast to **33**−**3a** and **33**−**4a**, the N-5 atom in the 6-acetyl-substituted **33**−**5a** is far away from the reactive position of the bound substrate. Therefore, **33**−**5a** shows low catalytic efficiency.

Both flavopapains **33**−**3a** and **33**−**4a** showed some trends in substrate specificity.[1] There was an increase in k_{cat}/K_M as the N-alkyl group of the dihydronicotinamide increased in chain length. This may be due to interactions between the N-alkyl substituents and the hydrophobic binding pocket of the flavopapains. It was also not surprising that NADH, having a relatively hydrophilic and bulky substituent, was a poor substrate for flavopapains **33**−**3a** and **33**−**4a**. Besides substrate specificity, **33**−**4a** showed significant but not complete stereoselectivity in its reactivity.[133] It exhibits a substantial (7-fold) preference for transfer the 4A (*pro*-R) hydrogen of NADH shown in Figure 35.

In addition, the semisynthetic flavopapain was also found to be able to discriminate between D and L enantiomers of various dihydronicotinamides in which the primary amide is substituted with an optically active α-methylbenzylamide side chain (**4a** and **4b, 5a** and **5b** in Figure 35).[136] The chiral selectivity between the D and L enantiomers was reported with the L isomer favored by approximately 2-fold. This detectable chiral discrimination indicates that the active site of flavopapain is asymmetric, as is required of an enzyme-like pocket. However, this selectivity is only modest and is insufficient for asymmetric synthesis applications.

Flavopapains were found to be able to mediate the oxidation of thiols to disulfides.[140,141] The generally accepted mechanism, shown in Figure 36, involves a rate-determining nucleophilic addition of a thiolate ion to the C-4A position of the flavin ring. The formation of this covalent adduct is assisted by general-acid catalysis at the N-5 position. Ionization of the remaining thiol followed by nucleophilic attack on sulfur generates the disulfide and reduced flavin anion.[142,143] The rates of oxidation of dithiothreitol (DTT), D,L-dihydrolipoic acid, and D,L-dihydrolipoamide catalyzed by flavopapain **33**−**4a** were quite

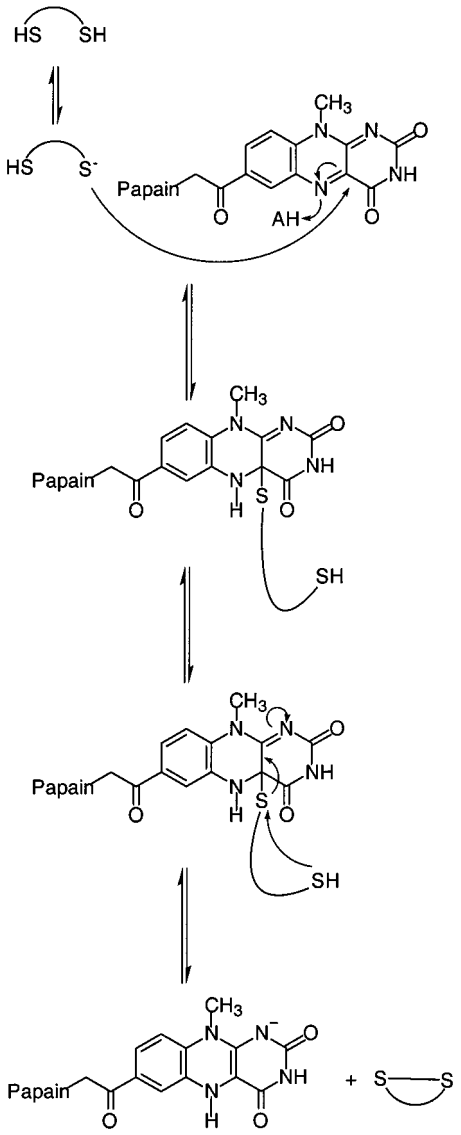


Figure 36. Mechanism of flavin-catalyzed oxidation of dithiols to disulfides.

modest.[140] The rate enhancements for oxidation of DTT and D,L-dihydrolipoic acid by flavopapain **33**−**4a** were 3.9- and 8-fold, respectively, when compared to the corresponding free flavin **33**−**4b**. The rate acceleration observed in these cases increases as the hydrophobicity of the substrate increases. In addition, replacing the charged carboxyl group of D,L-dihydrolipoic acid by a neutral amido moiety apparently stabilizes hydrophobic interactions between **33**−**4a** and the substrate. In contrast, flavopapain **33**−**3a** was found to be a significantly more effective catalyst for such thiol oxidation reactions.[141] Under anaerobic conditions, the values of k_{cat}/K_M for the oxidation of D,L-dihydrolipoamide and D,L-dihydrolipoic acid are 126 and 200 times higher than the second-order rate constants for the corresponding reactions with the model flavin **33**−**3b**. Unlike the stereoselectivities observed in oxidation of 1,4-dihydronicotinamide catalyzed by flavopapain, the oxidation of dithiols by flavopapain did not give similar results. The reason for the lack of stereoselectivity is probably related to the poor binding between these dithiol substrates and the flavopapins.

A second protein template that has been used for constructing semisynthetic flavoenzymes is glyceraldehyde-3-phosphate dehydrogenase (GAPDH).[144] In contrast to papain, active GAPDH is a tetrameric compound of four identical subunits (M_r 36 000). Each contains a binding site for both NAD^+/NADH and glyceraldehyde-3-phosphate. X-ray diffraction studies reveal that GAPDH contains an essential thiol functionality (Cys_{149}) in the active site. This sulfhydryl group is located near the binding site for the pyridine portion of NAD^+/NADH and is the putative binding site for glyceraldehyde-3-phosphate.[145,146] Importantly, the alkylation of Cys_{149} does not destroy the NAD^+/NADH-binding site.[147] Therefore, GAPDH was considered to be a suitable template for the design of a flavin-dependent oxidoreductase selective for NADH rather than for hydrophobic *N*-alkyl-1,4-dihydronicotinamides. GAPDH proteins from rabbit muscle tissue and from the thermophilie *Bacillus stearothermophilus* were used for the synthesis of flavo-GAPDH. Under aerobic conditions, the rate enhancement for the oxidation of NADH by 7-acetylflavo-GAPDH observed in the case of rabbit muscle enzyme was 83-fold and for the bacterial protein is nearly 6 000-fold.[144,148] Intriguingly, the bacterial flavo-GAPDH was stable at 55 °C. Under these high-temperature conditions (55 °C), the apparent bimolecular rate constant (k_p/K_s) was 56 200 M^{-1} s^{-1} while the second-order rate constant for the nonenzymatic model reaction increased to 23.1 M^{-1} s^{-1}. Hence, the net enzymatic rate acceleration at such a high temperature was still greater than 3 orders of magnitude.[148]

In contrast to the kinetic results obtained with flavopapain, both rabbit muscle and bacterial flavo-GAPDH show a different substrate preference (NADH > NADPH ≈ *N*-alkyl-1,4-dihydronicotinamide).[144,148] Such substrate specificity is understandable, given the known specificity of GAPDH itself,[145] and provides evidence that the flavin moiety was incorporated into the active site. The stereoselectivity of 7-acetylflavo-GAPDH was also studied for both rabbit muscle and bacterial flavoenzyme. Surprisingly, only the rabbit flavo-GAPDH exhibited a substantial preference for transfer of the *pro*-S hydrogen of NADH.[144] This is contrary to the result where transfer of the *pro*-R hydrogen was favored in the oxidation of the same substrate catalyzed by flavopapain. The reason only the rabbit muscle flavo-GAPDH had stereoselectivity was explained by the fact that the rabbit muscle flavoenzyme is active primarily as a tetramer whereas the bacterial flavoenzyme is dimeric. When the NADH binds in the binding site, the geometry of the binding pocket in the tetramer exposes only the *pro*-S hydrogen to the nearby flavin. The absence of complete stereoselectivity observed for the rabbit muscle flavo-GAPDH indicated that more than one reaction geometry was accessible. The lack of absolute stereoselectivity may be also due to the relative instability of the rabbit muscle protein.

More recently, in an effort to replace the P-450 reductase (a flavoenzyme) in the electron-transport system, a flavin moiety was covalently attached to

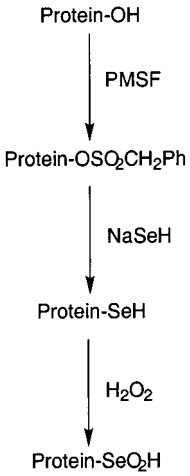


Figure 37. Semisynthetic synthesis of selenosubtilisin.

hemoglobin in the vicinity of the heme.[149] The chemical modification was carried out by reaction of a derivative of 7-cyanoisoalloxazine with the thiol group of cysteine β-93. This residue is located adjacent to the histidine β-92 which is coordinated to the heme iron. Two flavin moieties were introduced into each hemoglobin molecule (a $\alpha_2\beta_2$ tetramer). The resulting flavohemoglobin serves as a hydroxylase for aniline, obviating the need for the P-450 reductase. The rate acceleration for hydroxylation of aniline was dependent on the concentration of both aniline and NADPH, similar to the kinetic behavior of two-substrate enzyme reactions. The kinetic parameters for hydroxylation of aniline were K_M(aniline) = 5.5 mM and K_M(NADPH) = 0.22 mM. Moreover, the k_{cat} value (0.26 min^{-1}) was comparable to the corresponding values observed for microsomal cytochrome P-450 in the hydroxylation of aniline ranging from 0.22 to 0.65 min^{-1}.[150,151] These results indicate that the rate of the hydroxylase activity of flavohemoglobin is similar to that of microsomal cytochrome P-450. By comparing the kinetic parameters of three hydroxylase systems, flavohemoglobin, ferrihemoglobin (Hb^{3+}) with NADPH-cytochome P-450 reductase, and Hb^{3+} without P-450 reductase it was found that the apparent K_M(aniline) of the three systems was almost identical. In contrast, the apparent k_{cat} of Hb^{3+} without the P-450 reductase was much smaller than that of Hb^{3+} reconstituted with the P-450 reductase. Intriguingly, the apparent k_{cat} of the flavohemoglobin system is larger than that of the Hb^{3+} P-450 reductase system. This indicates that electron transfer between the neighboring prosthetic groups in flavohemoglobin proceeds even more efficiently than that in the combined Hb^{3+}−reductase system. Clearly the covalent attachment of flavins to several proteins has produced a plethora of interesting catalysts.

3. Selenium-Containing Systems

Inspired from earlier work on thiolsubtilisin and also from the unique chemistry of selenium, Wu and Hilvert[81] prepared selenosubtilisin using a two-step protocol, analogous to the method previously reported for making thiolsubtlisin. The hydroxyl group of Ser_{221} of subtilisin Carlsberg was selectively activated with phenyl methanesulfonyl fluoride (PMSF) to form a sulfonylated enzyme, which was treated with NaSeH and subsequently oxidized to the seleninic acid as shown in Figure 37.

Like thiolsubtilisin, selenosubtilisin was also a "damaged" protease but was a good acyl transferase. For example, the transfer of the cinnamoyl group to butylamine rather than water was 14 000 times more efficient for selenosubtilisin than for native subtilisin and more than 20 times more efficient than for thiolsubtilisin as shown in Figure 38.

In light of the interest in naturally occurring selenoenzymes, the redox properties of selenosubtilisin were investigated.[152] Like glutathione peroxidase, selenosubtilisin catalyzes the reduction of alkyl hydroperoxides by thiols. The reduction of *tert*-butyl hydroperoxide (t-BuOOH) by 3-carboxy-4-nitrobenzenethiol catalyzed by selenosubtilisin occurred at least 70 000-fold faster compared to the same reaction catalyzed by diphenyl diselenide, a well-studied antioxidant. The selenosubtilisin-catalyzed reaction proceeds through a ping-pong mechanism with at least one covalent intermediate as described in Figure 39.[153]

In analogy with the natural peroxidases, a variety of hydroperoxides were accepted as substrates for the semisynthetic enzyme while the dialkyl compound *tert*-butyl peroxide was not.[153,154] Kinetic investigation revealed that k_{max} was dependent upon the nature of the hydroperoxide, indicating that peroxide-medi-

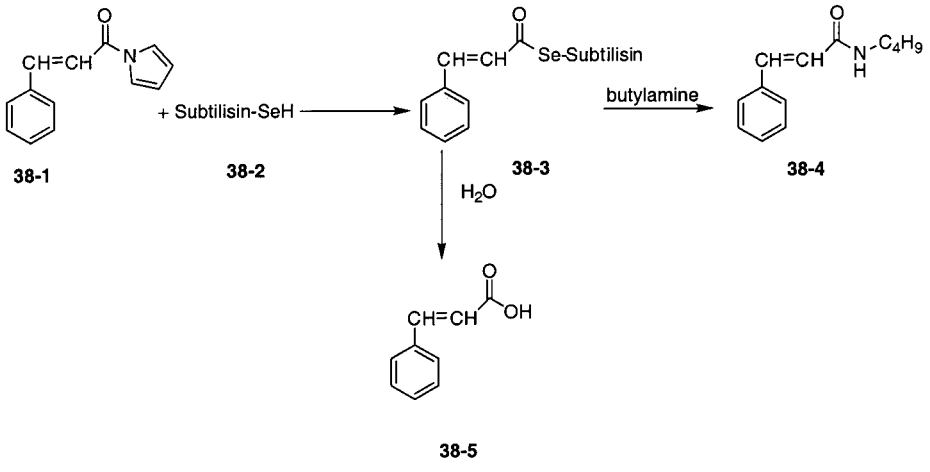


Figure 38. Acyl transfer reactions catalyzed selenium−subtilisin

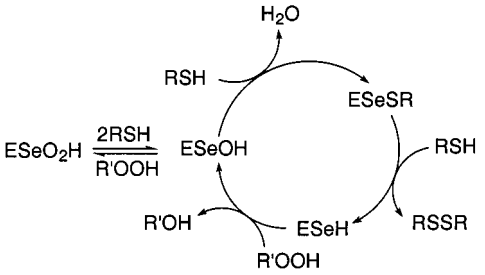


Figure 39. Mechanism of reactions catalyzed by selenosubtilisin.

ated oxidation of the enzymatic selenolate was at least partially rate-limiting. Crystallographic analysis of the semisynthetic enzyme[155] indicated that although the seleninic acid replaced the essential nucleophile in the enzyme's catalytic triad and introduced a negative charge into the active site, the interaction between His_{64} and Asp_{32} was not altered by the modification. Thus, this chemical modification did not disturb the overall structure of the protein or the catalytic triad, indicating the viability of chemical modification strategies for incorporating site-specific changes into the protein backbone.

Previous X-ray analysis and substrate screening with subtilisin suggested that its enantioselectivity could be described by a simple empirical binding model as shown in Figure 40.

Since selenosubtilisin has the same substrate-binding pocket as subtilisin, it was possible to rationalize and even predict its substrate selectivity.[8,156−158] A series of structurally varying racemic hydroperoxides was chemically synthesized and subjected to a kinetic resolution catalyzed by selenosubtlisin according to Figure 41. As a result and summarized in Table 4, all alkyl aryl hydroperoxides showed an enrichment of the enantiomers which had not previously been accessible.

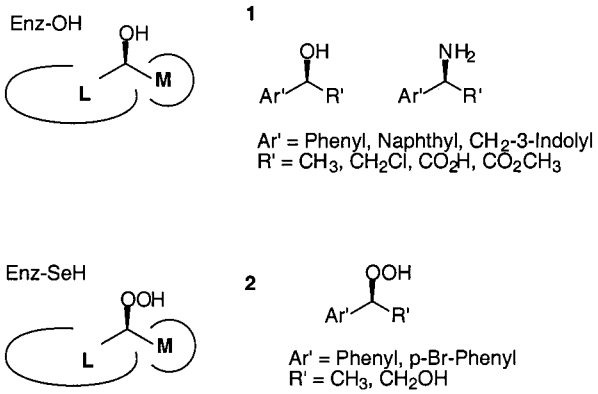


Figure 40. Empirical binding model of subtilisin and selenosubtilisin: L, large, hydrophobic residues; M, medium or polar groups. Some examples of preferred enantiomers in part **1**, the subtilisin-catalyzed esterification or acylation of racemic alkyl aryl alcohols or amines, respectively, are compared to part **2**, the preferred enantiomers in the seleno−subtilisin-catalyzed reduction of alkyl aryl hydroperoxides.

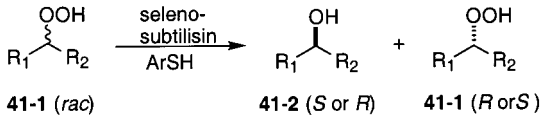
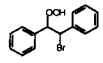


Figure 41. Kinetic resolution of alkyl peroxide by seleno−subtilisin.

The catalytic efficiency of the reactions was of the same order of magnitude as the native horseradish peroxidase. Highest selectivity (99% ee) was reached with substrate **41**−**1c**, which has a hydrophobic phenyl substituent on one side and a polar hydroxyl functionality on the other side of the hydroperoxy group. This arrangement fit exactly into the active site of selenosubtilisin, which is dominated by a large hydrophobic binding pocket (S_1) and a smaller more polar one (S_1'). For preparative advantages, the

Table 4. Results of Kinetic Resolution Reactions Catalyzed by Selenosubtilisin

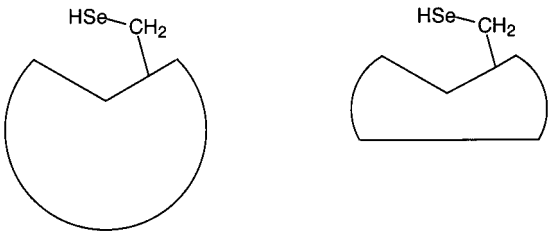
Hydroperoxide		Peroxide **40-1** ee (%)	Alcohol **40-2** ee (%)	K_M [mM]	k_{cat} [min^{-1}]	k_{cat}/K_M [$mM^{-1}min^{-1}$]
40-1a		52 (R)	60 (S)	15.7	2125	135
40-1b		34 (R)	28 (S)	4.3	592	138
40-1c		99 (S)	99 (R)	2.1	2443	1150
40-1d		64	90	0.07	3322	47500
40-1e		-	-	9.3	905	96
40-1f		4	4	1.8	33	19
40-1g		80	96	8.5	820	97
40-1h		14 (S,S)	30 (R,R)	5.3	449	84
40-1i		22 (R,R)	38 (S,S)	12.2	643	53
40-1j		60	44	-	-	13

selenosubtilisin exhibited the opposite sense in the enantioselectivity compared to the native peroxidases, which may complement the set of naturally available biocatalysts for enantioselective synthesis.

To improve the stability of selenosubtilisin, subtilisin was cross-linked using glutardialdehyde prior to seleno-modification of the active site Ser_{221}.[159,160] The final cross-linked crystals (CLC) of selenosubtilisin revealed similar kinetic properties to those of non-crosslinked peroxidase and very high stability under denaturing conditions. Since CLC-selenosubtilisin is insoluble in aqueous buffer as well as in organic media, it can be easily recovered after synthesis either by centrifugation or filtration. What is noteworthy is that the CLC-enzyme maintained high levels of activity and selectivity over 10 reaction cycles.

In a similar fashion as for constructing selenosubtilisin, Liu and co-workers[161] prepared a selenium-containing trypsin by converting the active site serine into selenocysteine. The glutathione peroxidase (GPX) activity of the seleno-trypsin is 40-fold less than that of native GPX, suggesting that glutathione (GSH) is not a particularly good substrate. However, this showed that it is viable to convert an active serine into selenocysteine in other serine proteases.

Catalytic antibodies raised against substrate-based analogue haptens provide another promising avenue for obtaining high affinity and specificity for the targeted substrate. Chemical modification of the antibody combining site residues (which are located at the variable regions) has the advantage of enabling the introduction of desired function in a well-defined way. A selenium-containing catalytic antibody (Se-4A4)[162] was prepared by converting one of the reactive serine residues per Fab fragment of a monoclonal antibody (4A4) raised against a GSH derivative into selenocysteine shown in Figure 42 (**42**−**1**).[163]



42-1 **42-2**

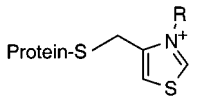
Figure 42. Schematic representation of catalytic antibodies with imidazole at the antibody-combining site: **(42**−**1)** selenium-containing catalytic antibody, **(42**−**2)** selenium-containing F_v catalytic antibody.

The chemically modified catalytic antibody acted as a glutathione peroxidase (GPX) mimic with a k_{cat}/K_m value of (9.4×10^6)-fold higher than the uncatalyzed reaction and with a turnover number about 42% of that of the natural rabbit liver cGPX under similar conditions.

In an effort to minimize the high immunogenicity and lower the molecular weight, a monoclonal antibody 3H4 (IgM) was raised against a glutathione-based hapten, hydrolyzed by pepsin treatment to yield an Fv fragment with minimal antigen-binding properties.[164] Active serines in the binding site were converted to selenocysteine (structure **42-2**). This Fv catalytic antibody showed similar binding affinity for GSH as MAb 3H4. It exhibited a high GPX activity of the same order of magnitude as native GPX from rabbit liver and was 2500 times more than that of PZ51, the best GPX mimic with low molecular weight known to date.

4. Thiazolium Chemistry

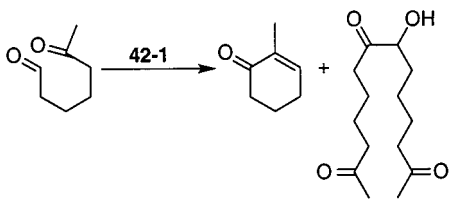
Suckling and Zhu[165] modified the active site cysteine of papain with different thiazolium analogues as shown in Figure 43.



43-1a R = $C_6H_5CH_2$
43-1b R = CH_3

Figure 43. Thiazolium−papain conjugates.

These thiazolopapains were devoid of the original protease activity but catalyzed the decarboxylation of pyruvic acid to form acetaldehyde.[166] Importantly, they also mediated carbon−carbon bond formation. Using 6-oxoheptanal as substrate, both the intra- and intermolecule carbon−carbon coupling shown in Figure 44 were observed.



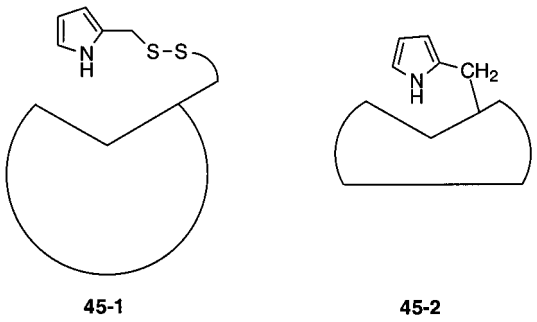
44-1 **44-2** **44-3**

Figure 44. Reactions catalyzed by thiazolium−papain.

Compared to the free coenzyme analogue, *N*-benzylthiazolopapain (**43**−**1a**) was effective at 400-fold lower concentrations and the yield was up to 5-fold higher under the similar conditions. In the reaction catalyzed by **43**−**1a**, 2-methylcyclohex-2-enone **44**−**2**, the expected product, was formed in 28% yield while the intermolecular product, 7-hydroxytetradecane-2,8,13-trione **44**−**3**, was obtained in 60% yield compared with a yield of 15% for **44**−**3** by the corresponding thiazolium salt lacking the protein component. The *N*-methylthiazolopapain **43**−**1b** proved to be less effective in mediating the reaction affording **44**−**2** and **44**−**3** in 8% and 20% yield, respectively. Significantly, these are the very first semisynthetic enzymes capable of the catalysis of carbon−carbon bond formation.

5. Catalytic Antibody Modification

Selective derivatization of active site residues can also be a way to introduce a wide variety of natural or synthetic catalytic groups in monoclonal antibodies. The antibody MOPC 315 binds substituted 2,4-dinitrophenyl (DNP) ligands with association constants ranging from 5×10^4 to 1×10^6 M^{-1}. [167]



45-1 45-2

Figure 45. Schematic representation of catalytic antibody with imidazole at the antibody combining site. **(45−1)** Imidazole moiety was introduced to the Fab fragment through a disulfide linkage. **(45−2)** Imidazole was introduced into the F_v fragment in the form of His using site-directed mutagenesis.

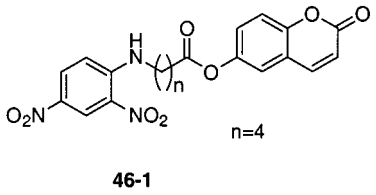
Imidazole was incorporated into this antibody via a thiol group introduced by chemically modifying Lys_{52H} in the active site via a disulfide linkage (structure **45-1**). Using the coumarin esters as the substrate, multiple (>10) hydrolytic turnovers were observed with no loss of activity. The catalytic efficiency, k_{cat}/K_m, for this reaction was about 1.1×10^3 times higher than that catalyzed by 4-methylimidazole.

For comparison, a hybrid Fv fragment of MOPC315 was constructed by reconstituting a recombinant variable light chain (V_L) produced in *E. coli* with a variable heavy chain (V_H) from the antibody.[168] Imidazole was introduced into the combining site by substituting Tyr_{34} residue of V_L with His using site-directed mutagenesis (structure **45-2**). This His mutant Fv catalyzed the hydrolysis of the 7-hydroxycoumarin esters of 5-(2,4-dinitrophenyl)-aminopentanoic acid **46−1** 90 000-fold faster compared to the reaction in the presence of 4-methyl imidazole at pH 7.8.

Using **46−1** as the substrate, Fv($Y34H_L$) turned over at least 11 times and still retained 44% of its activity. The loss of activity probably resulted from the accumulation of the inhibitory reaction product. The mutant Fv bound ϵ-2,4-DNP-L-lysine only 6-fold less tightly than wild-type protein, suggesting that the substituted Tyr residue is not involved in the DNP recognition process. Compared to the imidazole-catalytic antibody generated by tethering an imidazole chemically, a 16-fold greater rate increase was observed for the reactions catalyzed by His mutant Fv protein under similar conditions. This rate enhancement is likely due to the fewer degrees of the freedom of the imidazole moiety inside the combining site.

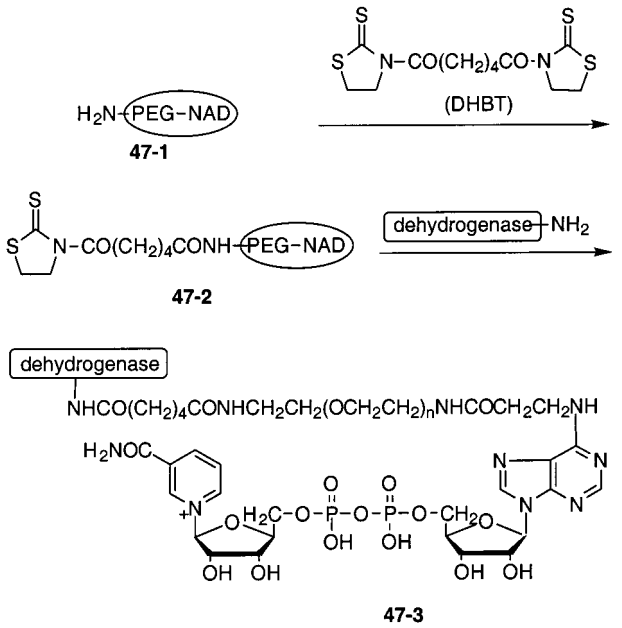
6. NAD^+ Conjugates

Semisynthetic NADH oxidases have been prepared by linking poly(ethylene glycol) derivatives of NAD (PEG−NAD) to a variety of dehydrogenases.[169] These constructs catalyze the transfer of electrons from NADH to oxygen or 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT).[169−173] Figure 47 shows the synthesis of such semisynthetic enzymes.[169,170] The N-terminus of PEG−NAD, **47−1**, was activated by acylation with an excess of 3,3′-(1,6-dioxo-1,6-hexanediyl)bis-2-thiazolidinethione (DHBT). The resulting activated PEG−NAD, **47−2**, was separated from the excess DHBT and covalently linked to a dehydrogenase to form conjugate **47−3**. Early studies using this approach describe the preparation of MDH−PEG−NAD^+ and GlcDH−PEG−NAD^+ by covalently attaching PEG−NAD to malate dehydrogenase (MDH) and glucose dehydrogenase (GlcDH). Intriguingly, a significant rate enhancement (10 000-fold) was observed for GlcDH−PEG−NAD^+ but not for MDH−PEG−NAD^+.[169,170]



46-1

Figure 46. Chromogenic 7-hydroxycoumarin esters used as substrate for imidazole-containing F_v catalytic antibodies.



47-1 47-2 47-3

Figure 47. Preparation of dehydrogenase-PEG−NAD conjugates.

Additional studies involved the incorporation of 5-thylphenazine (EP^+) into the aforementioned NAD conjugates. Several conjugates were synthesized, including EP^+−PEG−GluDH, EP^+−GlcDH−NAD^+, and EP^+−LDH−NAD^+.[172−174] The EP^+−PEG−GluDH conjugate had NADH oxidase activity; the EP^+ acts as a catalytic group which oxidizes NADH and the glutamate dehydrogenase functions as a substrate-binding site. The schematic catalytic cycle of EP^+−PEG−GluDH is illustrated in structure **48-1**.

Kinetic analysis of the NADH oxidase activity of EP^+−PEG−GluDH quantitatively shows the effect of the presence of the substrate-binding site near the catalytic group.[174] EP^+−GlcDH−NAD^+ and EP^+−LDH−NAD^+ conjugates were prepared by attaching both PEG−NAD and PEG−EP^+ to glucose dehydrogenase and lactate dehydrogenase, respectively. Structures **48-2** and **48−3** show the catalytic cycle of these oxidases (EP^+−GlcDH−NAD^+ and EP^+−LDH−NAD^+,

respectively).[173,174] The catalytic cycle of EP^+−GlcDH−NAD^+ starts with the reduction of the NAD^+ moiety. Then the NADH moiety is reoxidized by the ethylphenazine (EP^+) group by oxidation of glucose. Finally, the reduced ethylphenazine (EPH) is reoxidized by an electron acceptor (O_2 or MTT). Kinetic analysis of the EP^+−GlcDH−NAD^+-catalyzed reaction shows that this mechanism involving high effective concentration, coupling of successive catalytic reactions, and multiple connections between the two kinds of catalytic sites mimics the catalytic cycles of natural enzymes. EP^+−LDH−NAD^+ shows a different catalytic cycle (structure **48-3**) from that of EP^+−GlcDH−NAD^+.[174] There are two more enzyme forms containing an NAD^+ or NADH moiety in the coenzyme-binding site of the conjugate. Compared to glucose dehydrogenase, lactate dehydrogenase apparently has much higher activity and affinity for PEG−NAD^+.[169,175,176]

C. Protein and Enzyme Imprinting

A radical approach to protein engineering was developed by Keyes and colleagues. This strategy has four main components consisting of (1) partial or complete denaturation of a specific protein, (2) renaturation of the protein in the presence of substrate analogue or inhibitor, (3) chemical cross-linking of the resulting complex, and (4) removal of the substrate or inhibitor. The general idea is to select for protein conformations that have significant affinity for a particular substrate or inhibitor and trap these conformers by chemical cross-linking. For example, indole-3-propioic acid, a known inhibitor of chymotrypsin, was incubated with RNase A that was partially denatured at pH 3.0. This noncovalent complex was treated with glutaraldehyde followed by removal of the indole moiety. The resulting semisynthetic protein had esterase activity.[177,178] Other examples of this are outlined with minimal detail in the patent literature. Interestingly, this strategy has been employed to prepare fluorohydrolases that could be used to inactivate chemical warfare agents. In that work several enzymes and proteins were examined including BSA, casein, egg albumin, hexokinase, and RNase A. Using diisopropyl phosphoric acid as the templating substrate for RNase A renaturation, a semisynthetic fluorohydrolase was obtained that hydrolyzed diisopropyl fluorophosphate with a turnover number of 17.4 min^{-1};[179] multiple turnovers were reported for this catalyst. A more active form of this construct was subsequently reported.[180] Recently Liu et al. added an additional dimension to this strategy by combining it with the atom replacement methods described above.[181] In their approach, egg albumin was partially unfolded at pH 3.0 and imprinted with GSH-2DNP, a protected analogue of glutathione. After refolding at pH 8.0 and cross-linking with glutaraldehyde, serine residues adjacent to the glutathione-binding site were converted to selenocysteines via chemical modification with NaSeH. The resulting imprinted protein, an apparent dimer, displayed glutathione peroxidase activity (817 U/μmol) that was only 7-fold lower than glutathione peroxidase from rabbit liver (5780 U/μmol).

IV. New Frontiers

As noted above, the vast majority of protein modification reagents used to introduce or alter catalytic activity capitalize on the unique reactivity of the thiol group present in cysteine. While modification of other functional groups is possible, such approaches are rarely general in scope. One obvious limitation of thiol-based strategies is the lack of general methods to introduce single catalytic groups into proteins containing multiple cysteine residues. Recently, two new methods for protein modification that lack these limitations have been introduced. While neither of these has yet been used to prepare a semisynthetic enzyme, they are briefly discussed below.

In 1992, Perler and co-workers reported the presence of intein sequences in proteins that promoted their own self-excision—structural elements analogous to self-splicing introns present in RNA.[182−184] The mechanism of the protein excision reaction involves an activated thioester formed via intramolecular attack of an internal cysteine-derived thiol on the polypeptide backbone. Mutant intein sequences that do not allow the reaction to proceed following thioester bond formation have been identified. Such mutants allow large protein fragments possessing C-terminal thioesters to be easily obtained. Such activated species are valuable intermediates since they can be condensed with additional peptides or proteins carrying N-terminal cysteine residues via a process called "native chemical ligation".[185] This process is diagramed in Figure 49 in which the arrested splicing intermediate (**49**−**1**) undergoes a thiotransesterification reaction with a peptide to produce **49**−**2**. This intermediate then rearranges from the thioester to the more stable amide (**49**−**3**). An important feature of this approach is that it does not require a unique cysteine residue. Other non-N-terminal cysteines can undergo the transthioesterification reaction but are unable to form a new amide bond. In the context of semisynthetic enzymes, the key attribute of this process is that it can be used to introduce an enormous range of functionality into a protein. Essentially anything that can be incorporated into a peptide can be installed in a larger protein. This approach has been used by a number of groups for the introduction of various reporter groups and labels into proteins and promises to be a valuable technique for future semisynthetic enzyme construction.[186−188]

An alternative approach that addresses the "unique thiol" problem described above has been introduced by Tsien and co-workers.[189] Their approach, outlined in Figure 50, relies on the preparation of a rigid scaffold that positions two arsenoxides in register with two pairs of cysteines on the same face of a protein α-helix. The reagent they developed, denoted as FLASH-EDT_2 (**50**−**1**), reacts with a protein containing the sequence CCXXCC incorporated within a α-helical element (**50**−**2**) to produce a fluorescently modified protein (**50**−**3**). Since the sequence motif CCXXCC is uncommon, it can be introduced into desired target proteins to provide a unique site of attachment for FLASH-EDT_2 or other similarly designed reagents. While FLASH-EDT_2 will bind to

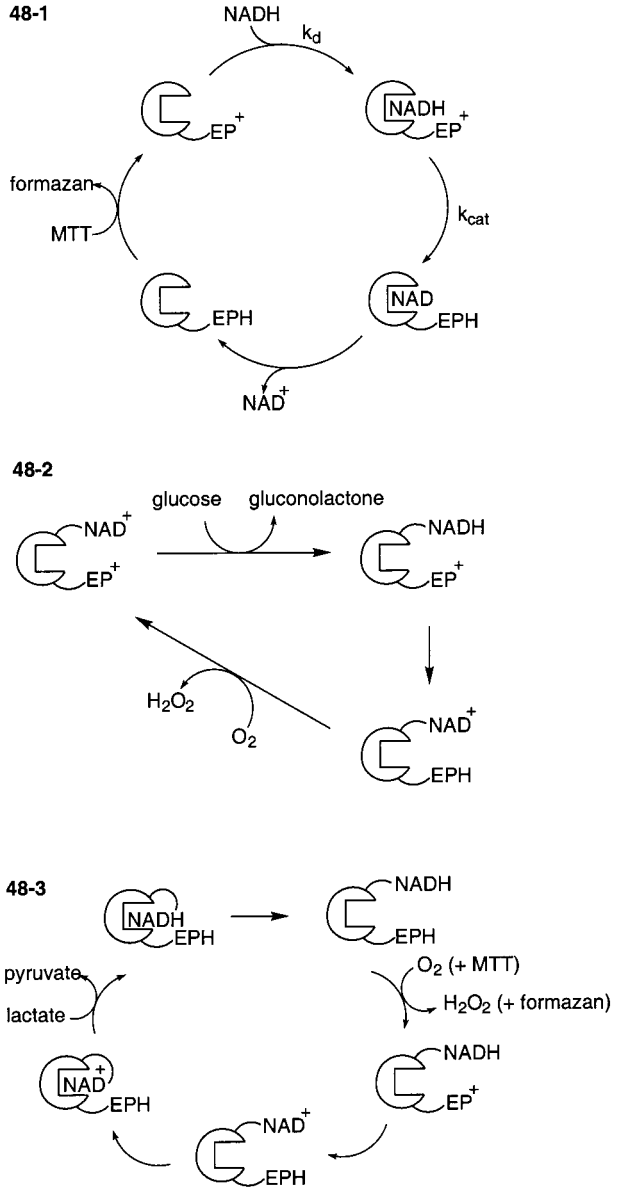


Figure 48. Catalytic cycles of (a) EP^+-PEG−GluDH, (b) EP^+−GlcDH−NAD^+, and (c) EP^+−LDH−NAD^+.

other sites within a protein with fewer cysteines, that nonspecific labeling can be minimized by the inclusion of exogenous thiols since the affinity of FLASH-EDT_2 for its cognate tetradentate site is greater than those with less optimal geometries or numbers of cysteines. One of the most useful features of FLASH-EDT_2 is that it is membrane permeable and reacts with its target site under in vivo conditions; with FLASH-EDT_2 it has been possible to label proteins within living cells. Thus, using reagents based on the arsenoxide chemistry employed in the design of FLASH-EDT_2 it may be possible to prepare semisynthetic enzymes in vivo. Such capabilities could facilitate a broad range of in vivo selection and evolution experiments that have not been possible with existing chemical modification technology.

V. Conclusions

This article describes approaches for chemically modifying enzymes and proteins to obtain catalysts

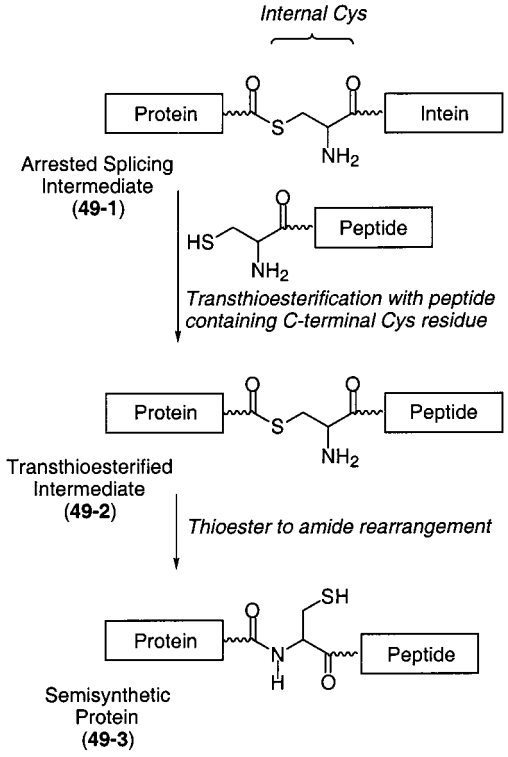


Figure 49. Strategy for introducing peptides into proteins using a protein-splicing intermediate and native chemical ligation.

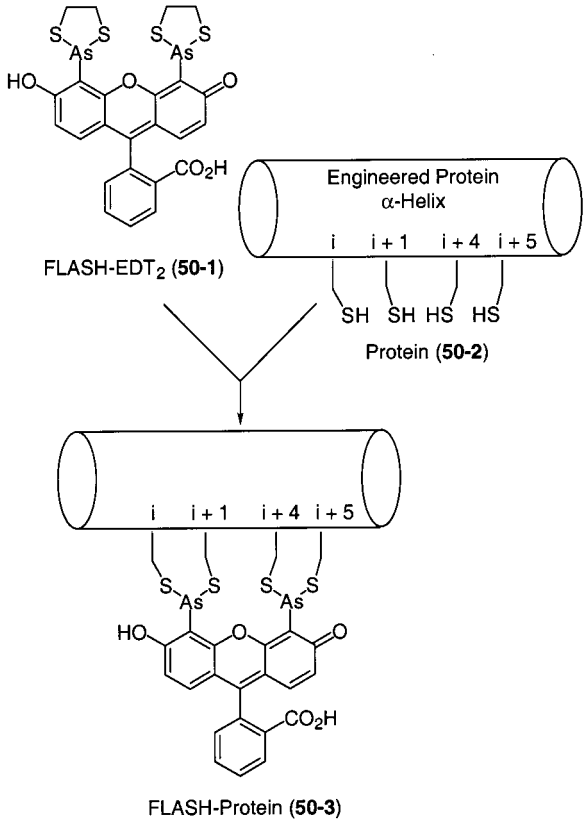


Figure 50. Introduction of FLASH-EDT_2 into a CCXXCC motif within a protein α-helix.

with either modified or completely new catalytic activities. Early work focused on chemical mutagenesis experiments in which a particularly reactive hydroxyl group in subtilisin or a thiol in papain was

used to obtain selective modification. This continues to be an active area of research. However, with the advent of site-directed mutagenesis, it is now possible to introduce cysteine residues that can be introduced at any desired location and subsequently functionalized with a wide range of reagents that can be used to both modulate existing enzymatic activity and create new enzyme-like catalysts. These studies have been aided by molecular modeling software packages that can be used for simple modeling experiments by nonexperts; such tools greatly facilitate experimental design and the interpretation of data. New synthetic methods that capitalize on native chemical ligation and intein-based strategies promise increased synthetic flexibility. While the catalytic efficiency of most of the semisynthetic constructs produced to date does not rival that of natural enzymes, significant rate accelerations have been obtained in some cases. Moreover, several covalently modified protein systems with catalytic activity have proved to be valuable probes for macromolecular structure. Given these demonstrable results together with the enormous potential of being able to introduce virtually any functionality into a protein, it is likely that covalent modification of proteins for biocatalyst development will continue to be an active area of research.

VI. Acknowledgments

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