8

Chemical Modification: Affinity Reagents

INTRODUCTION

A problem that is often encountered in chemical modification studies of the type described in Chap. 7 is limiting covalent modification to a few of the available side chains. An exception is where an unusually reactive side chain exists that can often be fairly specifically modified by reason of its more rapid reaction.

A second problem, also discussed in Chap. 7, is that side-chain-specific reagents may alter function by reaction at a site distant to the binding site or catalytic site being examined, leading to the erroneous conclusion that a certain residue is in or near that site.

The concept of site-specific labeling arose to circumvent some of these problems. The principle is quite simple: A reagent is synthesized that has characteristics of the ligand which usually binds to the site, but includes a reactive moiety that can react with residues in the binding site. Care is taken in the design of the reagent not to alter those ligand features that are essential for binding. Since such reagents have affinity for a specific site on the protein, they are usually referred to as "affinity reagents."

Once synthesized, the reagent (I^*), to be successful, must satisfy these requirements:

1. It must undergo reversible EI^* formation prior to irreversible inactivation. The existence of such complex formation is indicated by a saturable dependence of the rate constant for inactivation on the reagent concentration.

2. The extent of modification is limited relative to an unrelated reagent containing the same functional group when that group reacts readily with the protein. This is a result of effective concentration of the reagent. The affinity for the binding site gives a local concentration much higher than that in bulk solution, resulting in a more rapid modification of residues in the binding site than of similar residues elsewhere in the protein.

3. The modification can be protected by the presence of a competing, nonreactive ligand.

Before considering in more depth details of particular affinity reagents, we should complete our list of desirable characteristics of site-specific labels. As indicated, they should be structurally close to the parent compound and contain an active functional group that preferably reacts with a variety of different amino acid chains, and they should not be too bulky, so that steric problems do not arise. In addition to these three characteristics, site-specific reagents should produce stable derivatives of the modified protein in order that identification and quantitation of derivatized amino acid side chains is easy. As with many chemical modification reagents, availability of radio-labeled derivatives assists in quantitation and isolation of peptides. Finally, in the "ideal" reagent, one looks for such minor considerations as stability, solubility, and ease of synthesis or availability.

Affinity-labeling reagents have found increasing use in establishing the chemical nature of ligand binding sites in proteins. As we will see, in some cases it is possible to obtain information about a variety of amino acid residues in a particular site. This is achieved by the use of an affinity reagent with multiple reactivity or the use of a series of reagents with the reactive moiety at different positions in the molecule.

THEORETICAL CONSIDERATIONS

The formation of a reversible complex between the affinity reagent I* and its target protein is represented by

$$E + I^* \longleftrightarrow EI^* \longrightarrow E'I^* \tag{8-1}$$

where $K_d = [EI^*]/[E][I^*]$ and k is the rate of inactivation. The observed rate of inactivation at a given concentration of I*, K_{obs}, is given by

$$K_{obs} = \frac{k}{1 + K_d/[I^*]} \tag{8-2}$$

Therefore,

$$\frac{1}{K_{obs}} = \frac{1}{k} + \frac{K_d}{k}\frac{1}{[I^*]} \tag{8-3}$$

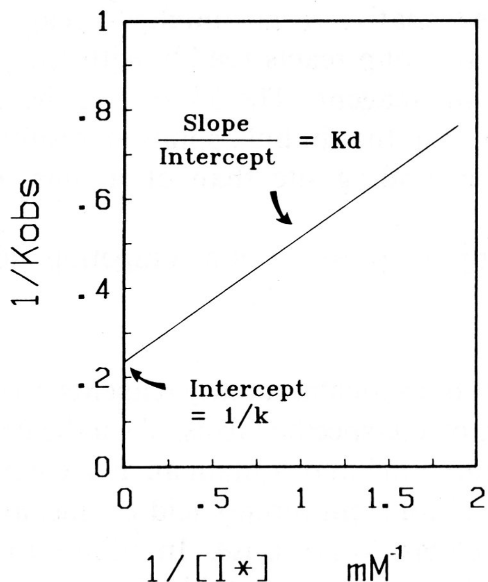


Figure 8-1 Determination of K_d and k for a functional site-specific reagent.

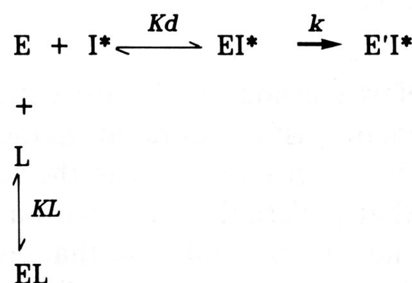


Figure 8-2 Scheme of competition between I* and L for protein binding sites.

indicating that a double-reciprocal plot, as in Fig. 8-1, allows determination of K_d and k.

One of the consequences of the formation of a reversible EI* complex is that protection against modification is afforded by analogs of I* that lack the reactive moiety. For such an analog (L), which forms a reversible EL complex as shown in Fig. 8-2, the dissociation constant K_L can be obtained from protection experiments.

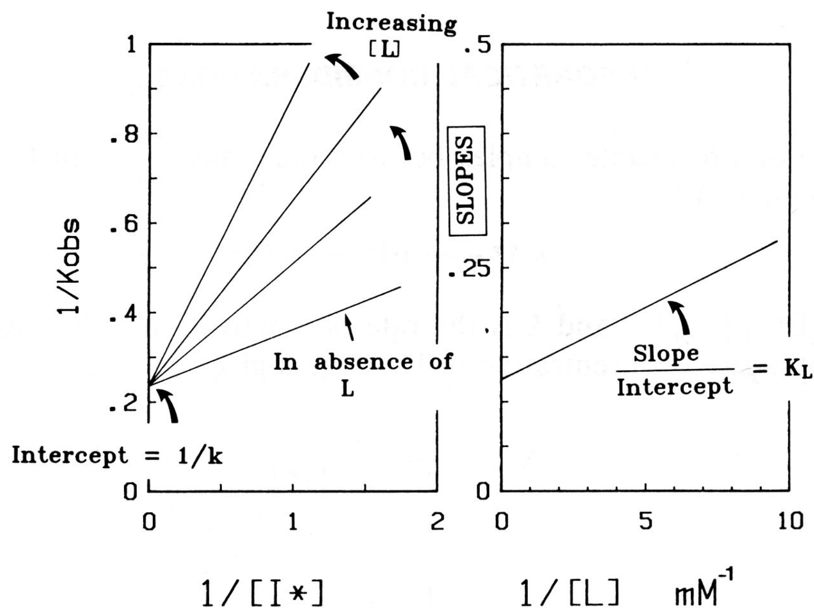


Figure 8-3 Double reciprocal plots for the determination of K_L from protection experiments.

By analogy with a simple competitive inhibitor we get

$$K_{\text{obs}} = \frac{k}{1 + (K_d/[\text{I}^*])}\left(1 + \frac{[\text{L}]}{K_\text{L}}\right) \tag{8-4}$$

and K_L as well as K_d, and k can be obtained from a series of determinations of the dependence of K_{obs} on $[I^*]$ at different concentrations of L, as shown in Fig. 8-3.

AFFINITY REAGENTS

Over the years many different types of site-specific reagents have been developed. Rather than attempt a comprehensive list, we examine one type in some detail since the considerations that go into their design and the problems encountered in their use are common to most site-specific reagents. The group we examine, because of their wide and varied uses, are the purine nucleotide analogs.

There are two classes of affinity reagents that can be considered. Some ligands, as part of their normal structure, contain a moiety that can be chemically converted to the required reactive group without a major perturbation of the structure of the ligand. We refer to them as endo-affinity reagents. In many cases, however, it is not possible to convert some part of the normal ligand to produce an affinity reagent, and a reactive moiety must be added to give an exo-affinity reagent. With exo-affinity reagents, particular attention must be paid to whether or not the additional moiety may interfere with or alter the normal binding specificity of the ligand.

Endo-affinity Reagents

The first class of purine nucleotide analogs that we consider are those arising from periodate oxidation. If we consider ATP as the parent compound, periodate cleavage results in oxidation of the ribose to give a 2′,3′-*cis*-dialdehyde (*O*-ATP, Fig. 8-4). This derivative, via the aldehyde moieties, will react primarily with lysine (or possibly N-terminal) amino groups, although a possible reaction with the sulfhydryl of cysteine has been suggested (Fig. 8-5).

If the amino group reacts to give a Schiff's base, reduction with sodium borohydride will give irreversible inactivation. Reduction with borotritiide will result in tritium labeling of the derivative. Such a scheme and results have been found in many enzymes with periodate-oxidized ATP, ADP, GTP, and even NADP (which can be enzymatically reduced to give *O*-NADPH).

In some enzymes, however [e.g., isocitrate dehydrogenase (ICDH)], labeling patterns obtained with radio-labeled reagents are inconsistent with the Schiff's base mechanism just mentioned. When ICDH is labeled with either *O*-ADP containing ^{14}C at the C-8 position, or with ^{32}P in the phosphates, one gets enzyme derivatives with different amounts of ^{14}C or ^{32}P labeling. In addition, reduction with sodium borotritiide results in *no* significant ^{3}H labeling. Such results have been explained in terms of the formation of a 4′,5′-didehydro-2′,3′-dihydroxymorpholino derivative after

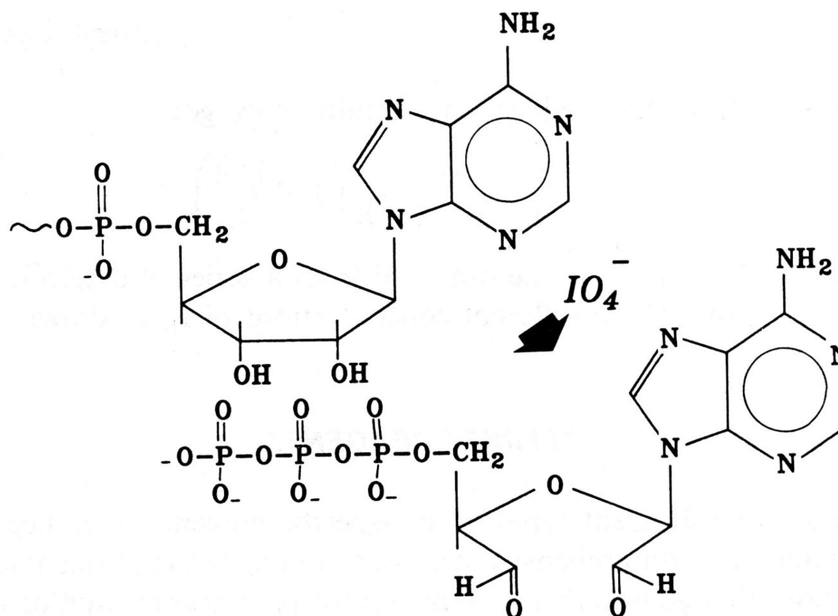


Figure 8-4 Periodate-oxidized ATP (*O*-ATP).

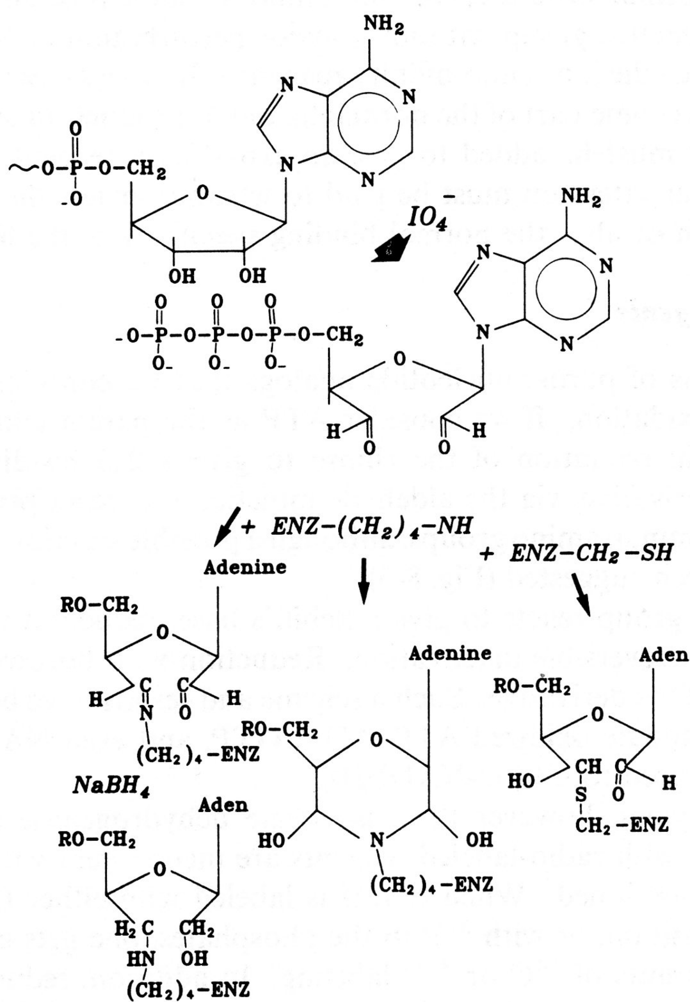


Figure 8-5 Possible reactions of periodate oxidized ATP with amino acid side chains.

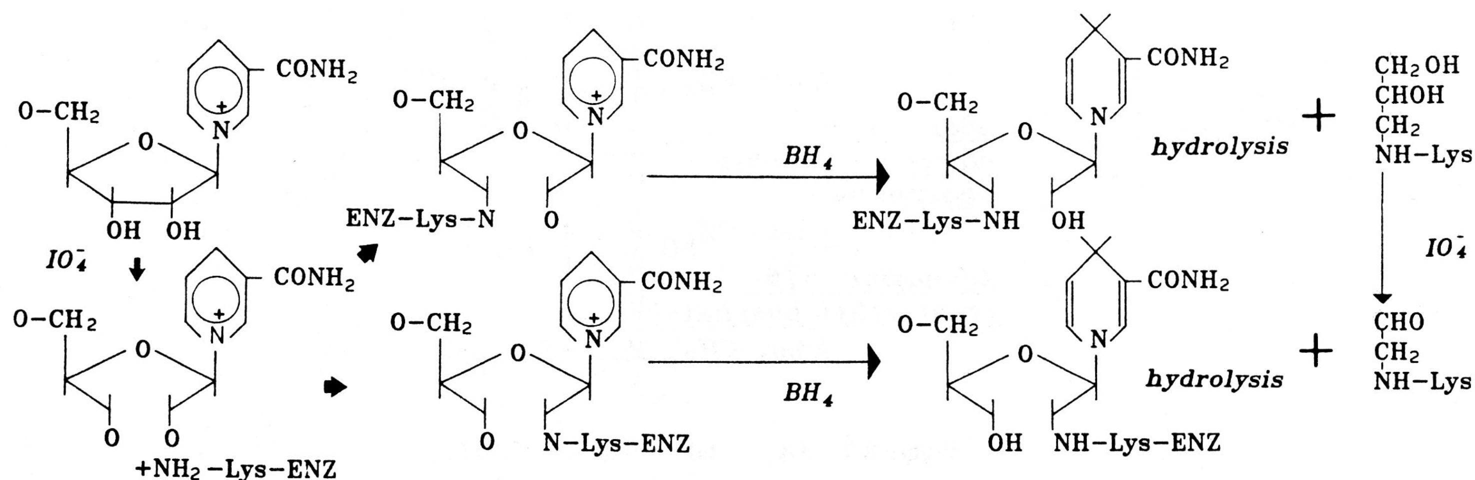


Figure 8-6 Reaction scheme for *O*-NADP.

reaction with the lysine amino group. Such products tend to be unstable to acid hydrolysis and subsequent amino acid analysis. The Schiff's base derivatives, however, are stable to hydrolysis to the extent that they give modified lysine derivatives. Shown in Fig. 8-6 is a reaction scheme for the inactivation and isolation of derivatized lysine for an enzyme modified by *O*-NADP. As can be seen, depending on which aldehyde in the *O*-NADP the Schiff's base is formed with, two derivatives of lysine can be obtained, which after periodate oxidation of the amino acid hydrolysate give only derivative II.

If we consider how periodate-cleaved purines rate as site-specific reagents in terms of our earlier criteria, we find that for structural considerations they rate well since only the ribose is modified and the modification does not involve large substituents. Many proteins that bind purines will tolerate alterations in the ribose ring. However, these derivatives do have drawbacks. The 2′,3′-dialdehyde reacts almost exclusively with lysine side chains rather than the more desirable case with wider side-chain specificity. In addition, when the reaction does not involve a Schiff's base the derivatives are somewhat unstable to acid hydrolysis or some of the harsher procedures that might, of necessity, be used in fragmentation.

Certain *alkyl halide derivatives* can also be considered endo-affinity reagents. This group can be regarded as having iodoacetamide as the parent compound, which can react with a variety of nucleophilic side chains, including cysteine, histidine, lysine, methionine, and to a minor extent, glutamate and aspartate. The most common derivatives are linked through the phosphates, as in the case of adenosine-5′-chloromethanepyrophosphonate and adenosine-5′-(β-chloroethylphosphate), whose structures, in relation to ATP, are shown in Fig. 8-7.

Not all alkyl halide derivatives of purines can be considered endo-affinity reagents, but with these derivatives of ATP the structure sufficiently resembles that of ATP for them to be considered as such. These derivatives can be very useful for labeling ATP binding sites if the purine part of the molecule provides the specificity. This type of derivative is not possible with NAD(P), of course.

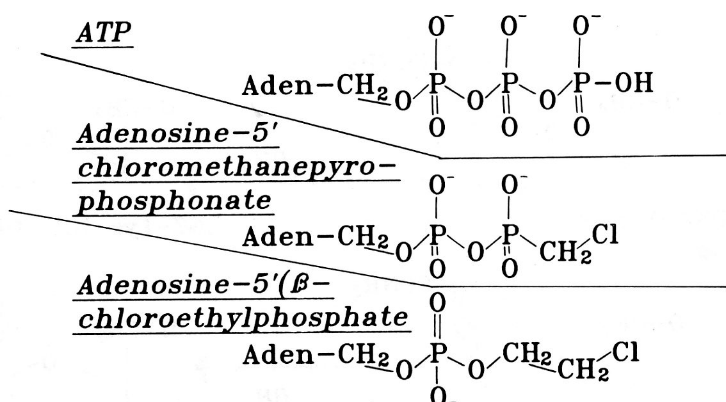


Figure 8-7 Alkyl halide derivatives of ATP.

Exo-affinity Reagents

The alkyl halide moiety is usually considered as producing an exo-affinity reagent since it is normally added to the ligand in such a way as not to interfere with binding. An example of such a use is the linkage of an alkyl halide derivative via the N-6 of the adenine, as in the case of *N*-6-*p*-bromoacetamidobenzyl ADP (Fig. 8-8).

In a most elegant extension of the ideas of site-specific labels, a series of new affinity labels that are based in part on the foregoing alkyl halide derivatives have been synthesized and shown to be effective. As already mentioned, a problem often encountered with affinity labels is a too stringent side-chain specificity. This has been partly overcome by the synthesis of purine nucleotide derivatives with *two* reactive moieties instead of the more usual one. These derivatives, whose structures are shown in Fig. 8-9, have the alkyl halide moiety *and* a dioxobutyl moiety built into the same six-position substituent, which for ease of synthesis has been replaced by a thio derivative.

These novel reagents have specificity for arginine in addition to the range of side chains with which the alkyl halides react. The halide reactivity is also increased by

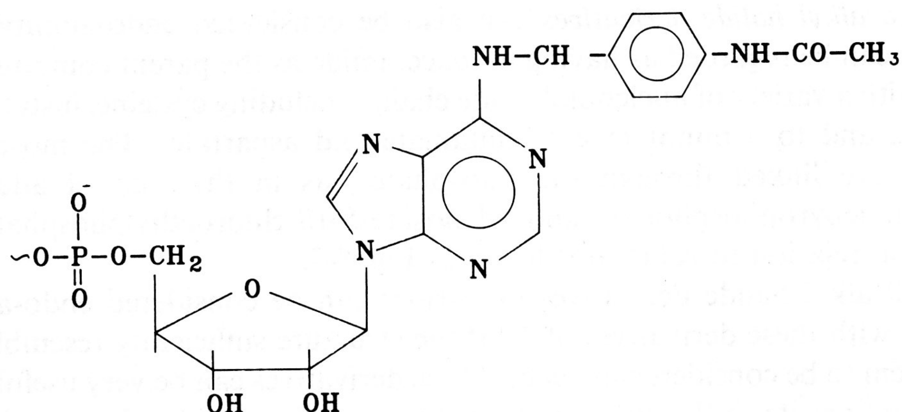


Figure 8-8 Structure of *N*-6-*p*-bromoacetamido-benzyl-ADP.

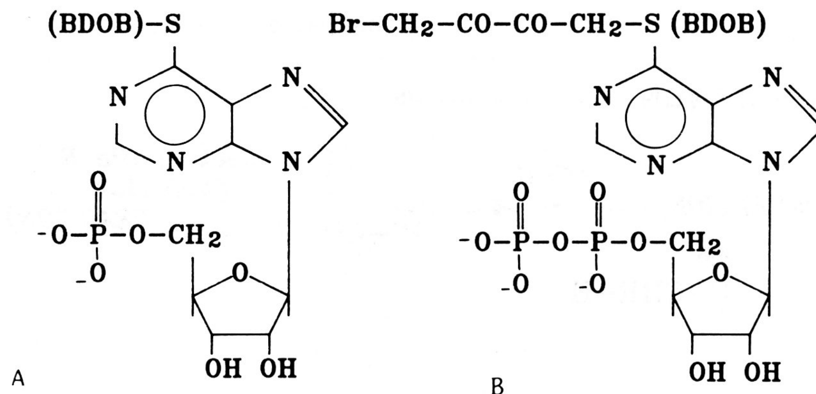


Figure 8-9 Structures of adenine nucleotide analogs: (A) 6-[(4-bromo-2,3-dioxobutyl)thio]-6-deaminoadenosine-5′-monophosphate; (B) 6-[(4-bromo-2,3-dioxobutyl)thio]-6-deaminoadenosine-5′-diphosphate.

having it in the form of a bromo-keto derivative, which helps overcome one inherent problem of the alkyl derivatives—low reactivity.

A different type of reactive group, which has been incorporated via the phosphate in both adenine and guanine nucleotides, is the fluorosulphonylbenzoyl (FSB) group found in adenosine 5′-(*p*-fluorosulphonylbenzoylphosphate), whose structure is shown in Fig. 8-10. These derivatives are particularly reactive toward lysine and tyrosine side chains. The FSB derivatives of ethanoadenosine have also been used to introduce a fluorescent group into proteins.

A special class of exo-affinity reagents are the photoaffinity reagents. In general, they have several distinct advantages over the other types of site-specific reagents:

1. They remain chemically inactive until they are activated (usually by light), which means that a wide variety of enzymic studies are possible without chemical modification occurring. In many cases the nonactivated derivatives are biologically active, allowing kinetic or regulatory properties of the derivatives to be established.
2. The generated group (diazo derivatives give a carbene, azido derivatives a nitrene: see Fig. 8-11) reacts fairly indiscriminately with a wide variety of side chains and thus does not suffer from being too side-chain specific.

There are, of course, problems, the most important of which is that the active species often has a quite long lifetime and as a result may dissociate from its binding site after activation but prior to labeling a residue in the binding site. Once in solution it may react nonspecifically with various side chains in the protein. Where such

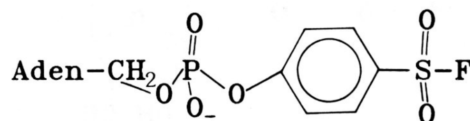


Figure 8-10 Structure of fluorosulfonylphenyl group.

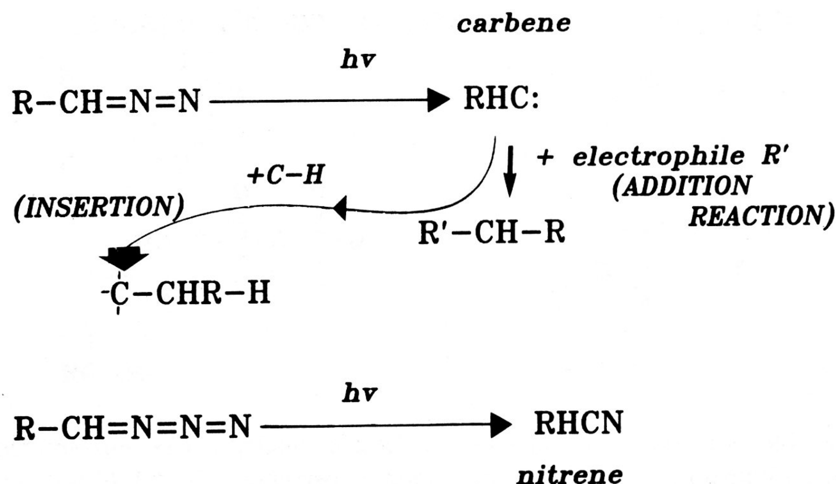


Figure 8-11 Generation of carbene or nitrene radicals in photo-affinity labels.

nonspecific labeling may present a problem (usually with long-lived active species that bind weakly to their specific binding sites), protection studies can distinguish nonspecific from specific labeling.

8-Azido derivatives (Fig. 8-12) have been used extensively; however, one problem that may be encountered is their tendency to adopt a syn conformation while the parent nucleotides exist predominantly in the anti conformation. Other derivatives, such as arylazido-β-alanine ATP or arylazido-β-alanine NAD(P), where the photo-affinity substituent is linked via ribose hydroxyls (Fig. 8-13), have been used successfully.

While the azido derivatives might be considered as endo-affinity reagents, the arylazido derivatives are clearly exo-affinity. These reagents have extremely bulky substituents, however, which can lead to two problems: (1) the substituent may interfere with binding—either preventing or distorting it, and (2) because of the distance between the reactive azido group and the major areas of the molecule involved in binding to its specific site, labeling may occur at adjacent residues rather than directly in the binding site.

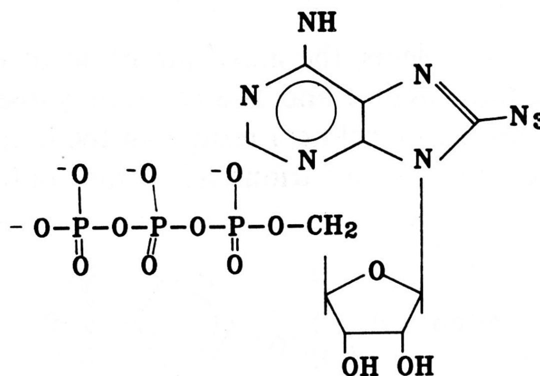


Figure 8-12 8-Azido adenosine triphosphate.

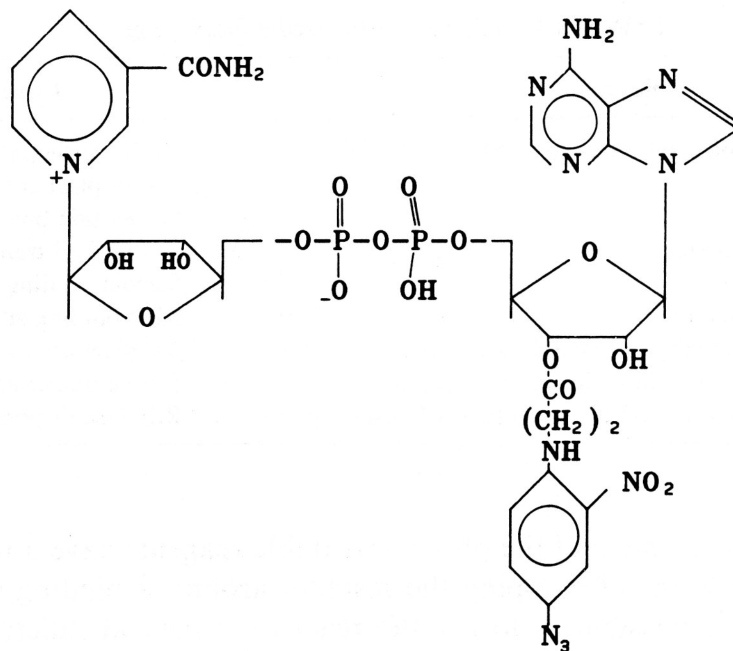


Figure 8-13 Structure of arylazido-β-alanine NAD.

This particular effect results in the type of data shown in Fig. 8-14 for modification of glutamate dehydrogenase by FSB-ethano-A, a supposed GTP-specific site reagent. The modified enzyme clearly is still responsive to the allosteric inhibitor GTP. The modification has occurred at some site adjacent to, rather than directly in, the GTP site, and caused decreased affinity rather than blocking GTP binding.

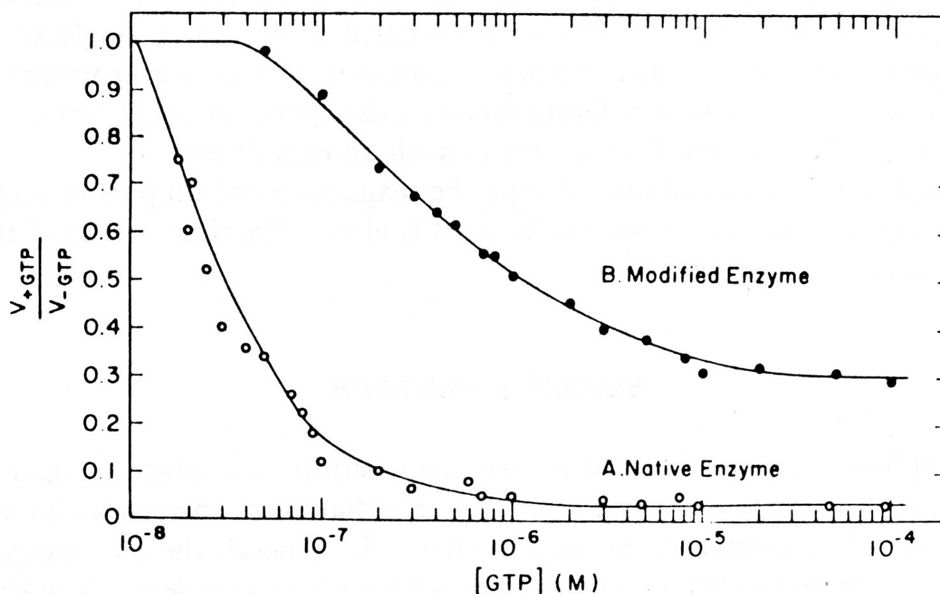


Figure 8-14 Inhibition of native and GTP-site "modified" glutamate dehydrogenase by GTP. (Reprinted with permission from: M. A. Jacobson and R. F. Colman, *Biochemistry*, *21*, 2177–2186. Copyright 1982 American Chemical Society, Washington D. C.)

TABLE 8-1 Selected synthesized affinity reagents

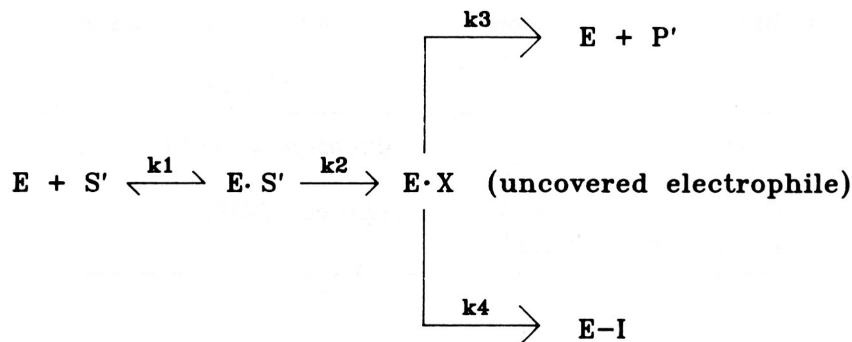
Reagent	Target enzyme
4,5-Dimethoxy-2-nitrobenzyl esters of cAMP or cGMP	Cyclic nucleotide binding proteins
2-Azido-ADP	Chloroplast coupling factor 1
Procion Blue MX-R	Nucleotide binding sites
6-Diazo-5-oxo-L-norleucine	γ-Glutamyl transpeptidase
2-Diazoestrone sulfate	Steroid binding sites
Bromoacetylpyridoxamine	PLP binding sites
β-D-Galactopyranosylmethyl-*p*-Nitrophenyl triazine	β-Galactosidase
9-(3,4-Dioxopentyl)hypoxanthine (reacts with arginine)	Purine nucleoside phosphorylase
2-(4-Bromoacetamido)anilino-2-deoxypentitol-1,5-bisphosphate	Ribulose bisphosphate carboxylase

Exo-affinity reagents and the photoactivatable reagents have a particularly useful application in terms of mapping the residues around a binding site. With exo-reagents it is often possible to locate the reactive moiety at different parts of the parent compound (provided that the affinity is not reduced to the extent that the advantage of an affinity reagent is lost). This allows the reactive moiety the chance to modify more than one residue in (or near) the binding site. The advantage is greatest with a photoaffinity label where there is little specificity of the generated carbene or nitrene. Exo-reagents, where the reactive moiety is freely mobile about its point of attachment to the parent compound, have the potential to label any reactive side chain within this sphere of influence. Analysis of the various residues that are labeled indicates which side chains are located in the vicinity of the binding site. If there is a chance that more than one binding site exists, protection experiments are of vital importance. All residues located at or near a single site will be protected *to the same extent* at a particular concentration of the protecting ligand. Where two or more sites with differing affinity exist, some modified residues will be protected to a greater extent than others in such an experiment.

Although we have considered site-specific reagents based on purine nucleotides, the list of reagents that have been synthesized is almost limitless. Some of the more interesting are listed in Table 8-1.

SUICIDE INHIBITORS

A suicide inhibitor is the product of an enzymic reaction on a substrate analog which produces a highly reactive enzyme-bound intermediate that chemically modifies the active site that has produced this intermediate. In general, the enzyme-generated reactive species is an electrophile that reacts with a protein nucleophile in the active site to give an irreversible covalent derivative. The scheme in Fig. 8-15 illustrates that there is always a competition, once the electrophile has been generated, between the inactivation event (k_4) and the dissociation of the electrophile prior to the modification event taking place (k_3).



$$k3/k4 = \textbf{Partition Ratio} = \frac{\textbf{No. of Product Molecules}}{\textbf{Inactivation Event}}$$

Figure 8-15 Scheme of a mechanism of a suicide inhibitor.

The ratio of k_3/k_4 is known as the partition coefficient for inactivation, and for an effective suicide inhibitor should be a low number. This is especially true where the generated active species may diffuse and modify enzyme nucleophiles other than those in the active site, leading to inactivation. Studies of the time course of inactivation allow a distinction to be made between these two cases. In true suicide inhibition, activity will in general be lost in a first-order process, while in the latter case loss of activity will follow an initial lag period. There are many ways that suicide inhibitors can be generated, but we consider only a few examples here.

Figure 8-16 illustrates the process by which the enzyme β-hydroxydecanoyl thiol dehydrase is activated by the acetylenic analog of its substrate. The enzyme catalyzes

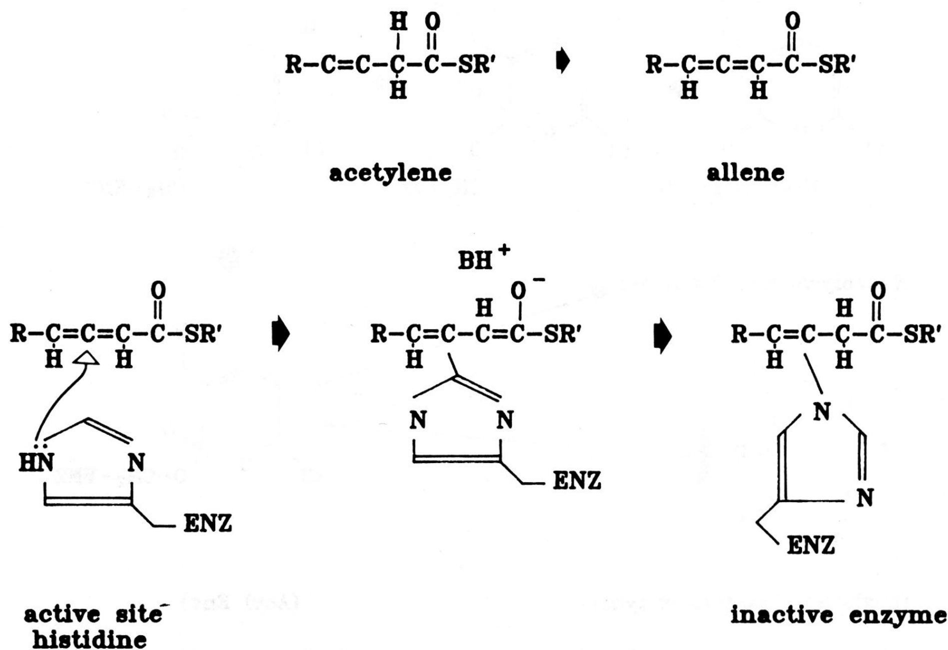


Figure 8-16 Suicide inhibition of β-hydroxydecanoyl thiol ester dehydrase.

TABLE 8-2 Enzymes subject to inhibition by acetylenic substrates

Enzyme	Inhibitor
Aromatase	10-Propargyl-4-ene-3,17-dione
GABA transaminase DOPA decarboxylase Prostaglandin synthase	α-Acetylenic DOPA

the isomerization of the unreactive 3-acetylenic moiety to a 2,3-allene conjugated to the thioester group, and it is this allene that reacts with an active-site histidine to give the inactive enzyme. Acetylenic substrate analogs have been widely used as suicide inhibitors in a number of systems, a few of which are shown in Table 8-2.

The serine proteases have been targets for suicide inhibitor design, and two basic approaches are employed. The enzyme can generate an intermediate which is so reactive that it leads to unwanted (by the enzyme) covalent modification.

Incubation of chymotrypsin with the substrate, benzyl chloropyrone, leads to suicide inhibition of chymotrypsin (with a partition ratio of about 20) by generation of the active acyl chloride moiety as a result of acyl-enzyme formation. The acyl chloride generated reacts with an enzyme nucleophile to give the inhibited enzyme, as shown in Fig. 8-17.

In the second strategy, which can be used where covalent intermediates such as the acyl-enzyme intermediate lie on the normal reaction pathway of the enzyme

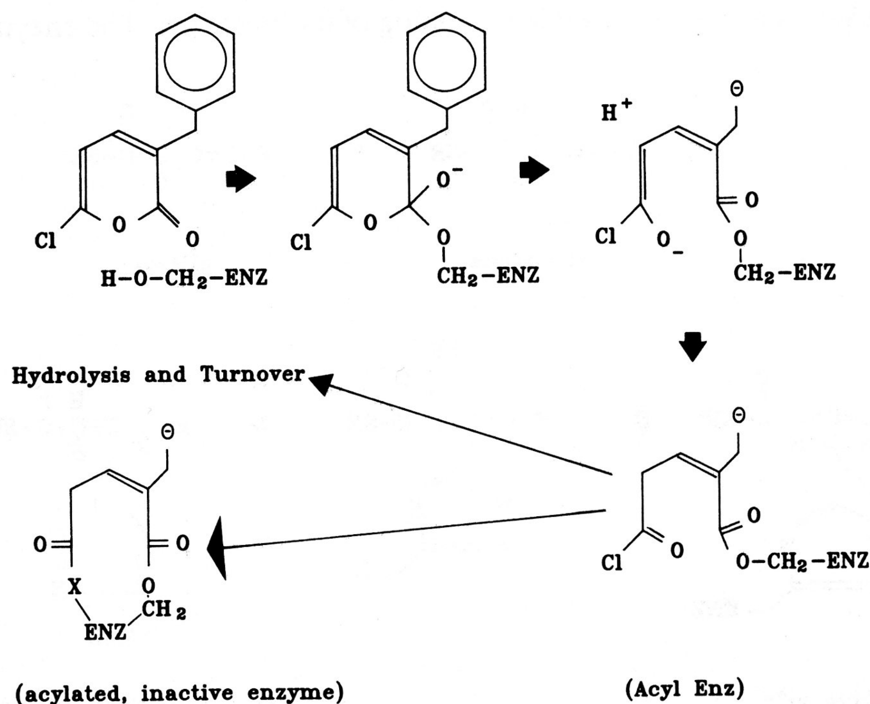


Figure 8-17 Suicide inhibition of chymotrypsin by benzoyl chloropyrone.

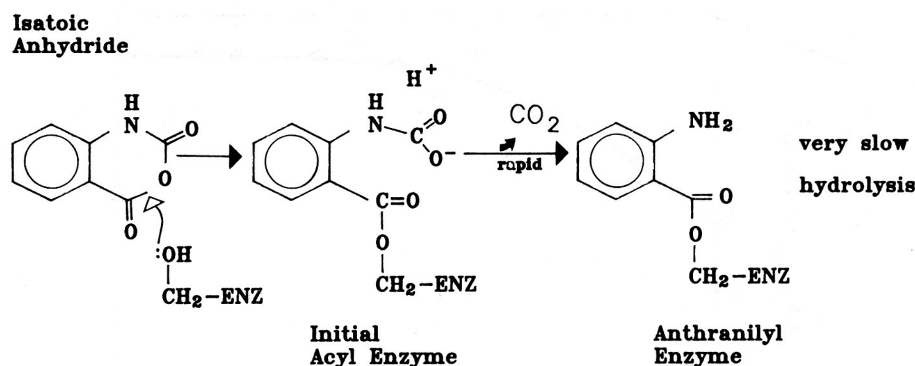


Figure 8-18 Suicide inhibition of chymotrypsin by isatoic anhydride.

mechanism, the substrate is modified so that the acyl-enzyme intermediate cannot break down to give product.

Chymotrypsin acts on isatoic anhydride (Fig. 8-18) to form an enzyme-acyl intermediate that in this case is rapidly hydrolyzed, not at the point of attachment to the enzyme, but at the generated carbamate, resulting in an anthranilyl enzyme intermediate that is hydrolyzed exceedingly slowly.

EXAMPLES OF SITE-SPECIFIC REAGENTS

Modification of Glutamate Dehydrogenase with 5′-p-Fluorosulfonylbenzoyl Adenosine (FSBA)

References: K. V. Saradambal et al., *J. Biol. Chem., 256*, 11866–11872 (1981).
J. A. Schmidt and R. F. Colman, *J. Biol. Chem., 259*, 14515–14519 (1984).

In a successful chemical modification experiment with a site-specific reagent a number of different experimental approaches must be used to establish that the reagent is indeed site specific prior to the identification of the modified residue or residues. The example of the modification of glutamate dehydrogenase by FSBA is a good one to examine since over a period of years the work has been taken to its logical conclusion: The residues modified by the reagent have been identified.

Kinetics and Quantitation of the Modification. With glutamate dehydrogenase, incubation with FSBA (at pH 8 in buffer containing 10% ethanol to assist solubility of the FSBA) results in a time-dependent *increase* in activity when the enzyme is assayed under conditions giving inhibition by excess NADH. This suggests that the site modified by FSBA is the inhibitory NADH site the enzyme is known to possess. As shown in Fig. 8-19, a semilog plot of the data is linear, allowing calculation of a rate constant of the modification. Protection studies show that an unrelated ligand, GTP, has no effect on the rate constant for modification, but NADH is an effective competitor of the modification.

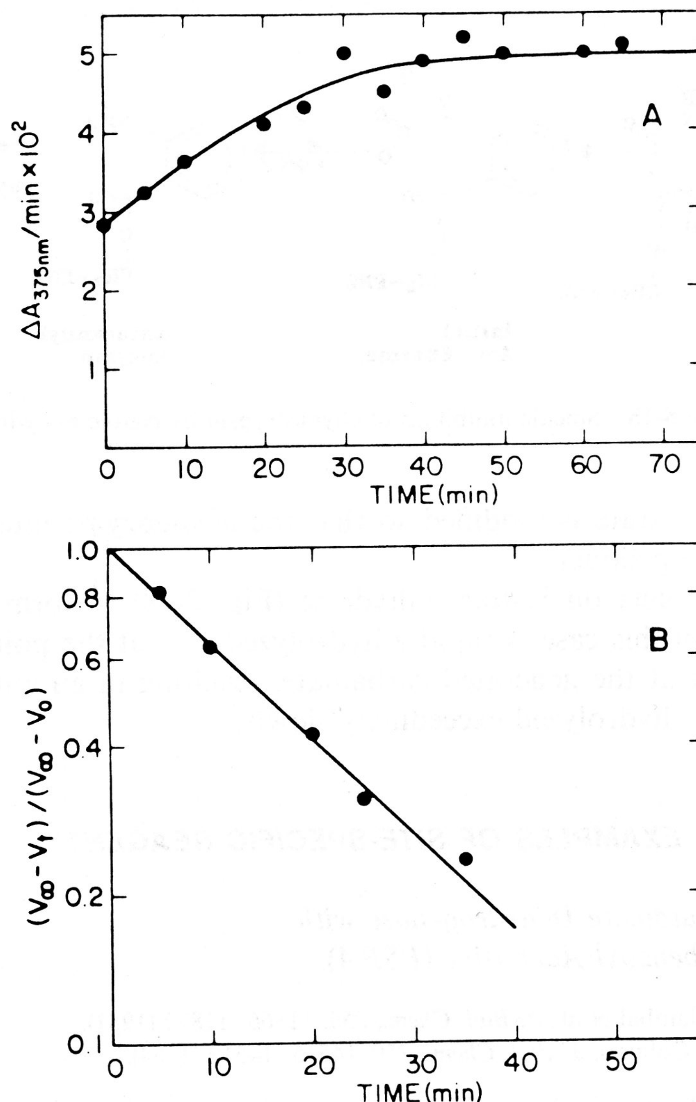


Figure 8-19 Reaction of GDH with FSBA: (A) rate of enzyme reaction of aliquots of enzyme withdrawn from an incubation mix containing FSBA and assayed at time intervals up to 70 minutes; (B) semilog plot of the data from part (A), where V_0 and V_t are the enzyme rates at $t = 0$ and any time t, and V_∞ is the velocity when the modification is complete. [From K. V. Saradambal, A. Bednar, and R. F. Colman, *J. Biol. Chem.*, *256*, 11,866–11,872 (1981). Reprinted with permission of the copyright owner, The American Society of Biological Chemists, Inc., Bethesda, Md.]

The reaction can be quantitated using radioactive FSBA, and Fig. 8-20 shows the percent change in NADH inhibition as a function of the number of moles of FSBA incorporated per subunit. The results clearly indicate that complete loss of NADH inhibition is the result of the modification of significantly *less* than one site per subunit. The possibility that such results could be due to a concentration-dependent aggregation of the protein masking some sites is eliminated by the observation that the stoichiometry of modification is unchanged at two protein concentrations.

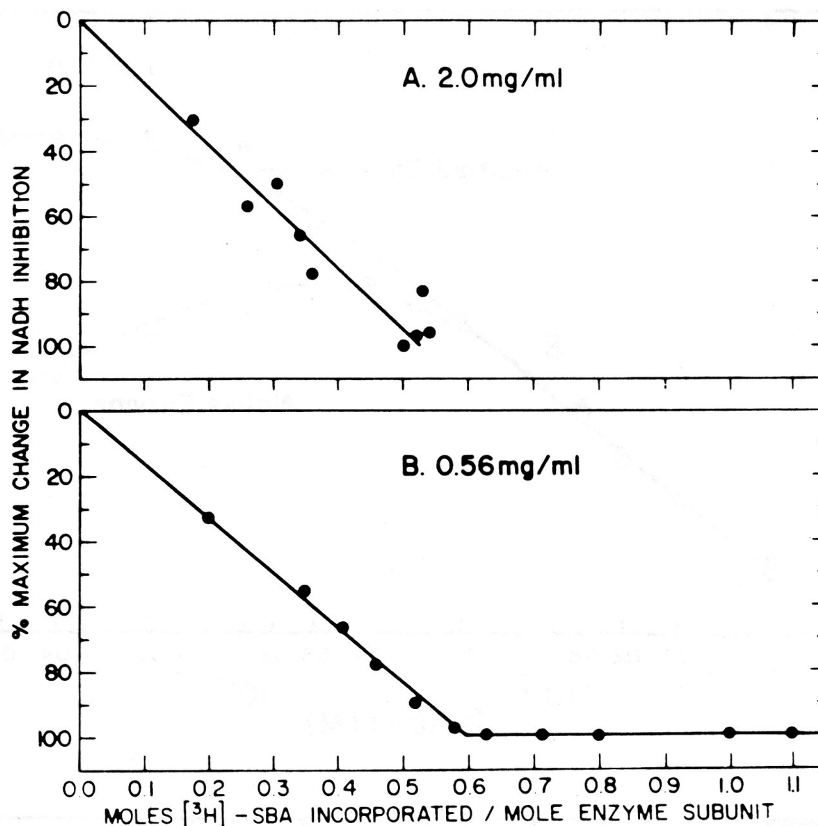


Figure 8-20 Quantitation of FSBA modification. [From K. V. Saradambal, A. Bednar, and R. F. Colman, *J. Biol. Chem.*, *256*, 11,866–11,872 (1981). Reprinted with permission of the copyright owner, The American Society of Biological Chemists, Inc., Bethesda, Md.]

Characterization of the Effects of Modification. Once the stoichiometry of modification is established, the kinetic properties of the modified enzyme are examined. The data in Fig. 8-21 show that the substrate inhibition seen at high NADH concentrations with the native enzyme is absent in the modified enzyme. Although in this particular example it was shown that the modified enzyme is still subject to inhibition by GTP, the various kinetic parameters of the enzyme were not examined.

Identification of Amino Acids Modified. The sulfonyl-fluoride moiety of FSBA is capable of reacting with a number of side chains in proteins, especially serine, cysteine, lysine, and tyrosine. In this example, modification of serine and cysteine is eliminated from further consideration since (1) after modification and acid hydrolysis no pyruvate could be detected (serine modification produces a dehydroalanine residue following incubation with sodium hydroxide which will release pyruvate on acid hydrolysis), and (2) the total cysteine residues in the protein, as determined by DTNB titration, were unchanged after modification.

After acid hydrolysis of protein modifed to varying extents with FSBA, carboxybenzenesulfonyl (CBS)-lysine and CBS-tyrosine, the expected acid hydrolysis products of modified lysine and tyrosine, are both identified in amino acid analysis.

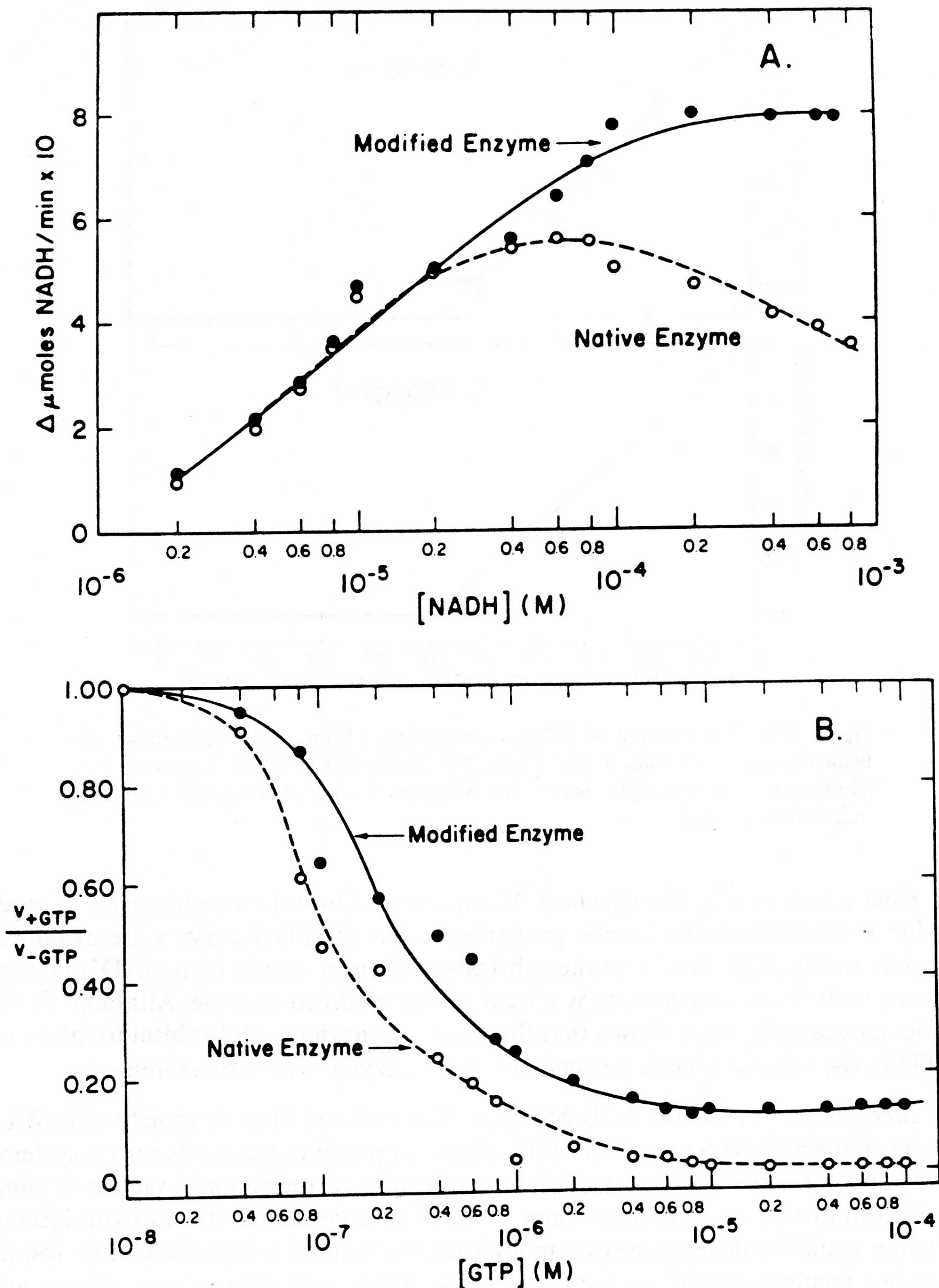


Figure 8-21 Comparison of kinetic properties of native and modified enzyme. [From K. V. Saradambal, A. Bednar, and R. F. Colman, 11,866–11,872 (1981). Reprinted with permission of the copyright owner, The American Society of Biological Chemists, Inc., Bethesda, Md.]

Furthermore, both residues are modified to approximately the same ratio no matter what the total incorporation of FSBA per enzyme molecule.

Location of Modified Residues in the Sequence. The modified protein is subjected to thermolysin proteolysis after denaturation (with urea), reduction, and carboxymethylation. After digestion the modified peptides are initially purified by affinity chromatography using dihydroxyboryl-substituted cellulose, which interacts reversibly with the *cis*-diol moiety of the modified residue. Two peptides are subsequently purified by HPLC and subjected to amino acid analysis. Since the entire amino acid sequence of glutamate dehydrogenase is already known, the amino acid analysis of the two modified peptides is sufficient to uniquely characterize the thermolysin fragments modified. The two modified residues are identified as tyrosine-190 and lysine-420.

β-Lactamase Suicide Inhibitors

References: D. G. Brenner and J. R. Knowles, *Biochemistry*, *20*, 3682–3687 (1981). C. J. Easton and J. R. Knowles, *Biochemistry*, *21*, 2857–2862 (1982). M. Arusawa and S. Adam, *Biochem. J.*, *211*, 447–454 (1983).

A variety of compounds that behave as suicide inhibitors of β-lactamase have been described. Sulbactam, whose reaction is shown in Fig. 8-22, results in a double

Figure 8-22 6,6,D_2-sulbactam as a suicide substrate of β-lactamase.

covalent modification, the acyl enzyme intermediate being stabilized by reaction of a generated electrophile with an enzyme nucleophile.

A somewhat different mechanism of suicide inhibition results from the use of Δ^2-pyroline; two forms of the acyl enzyme intermediate are formed, one of which, the Δ^2-acyl enzyme, is readily hydrolyzed, while the Δ^2-acyl enzyme is hydrolyzed very slowly (Fig. 8-23).

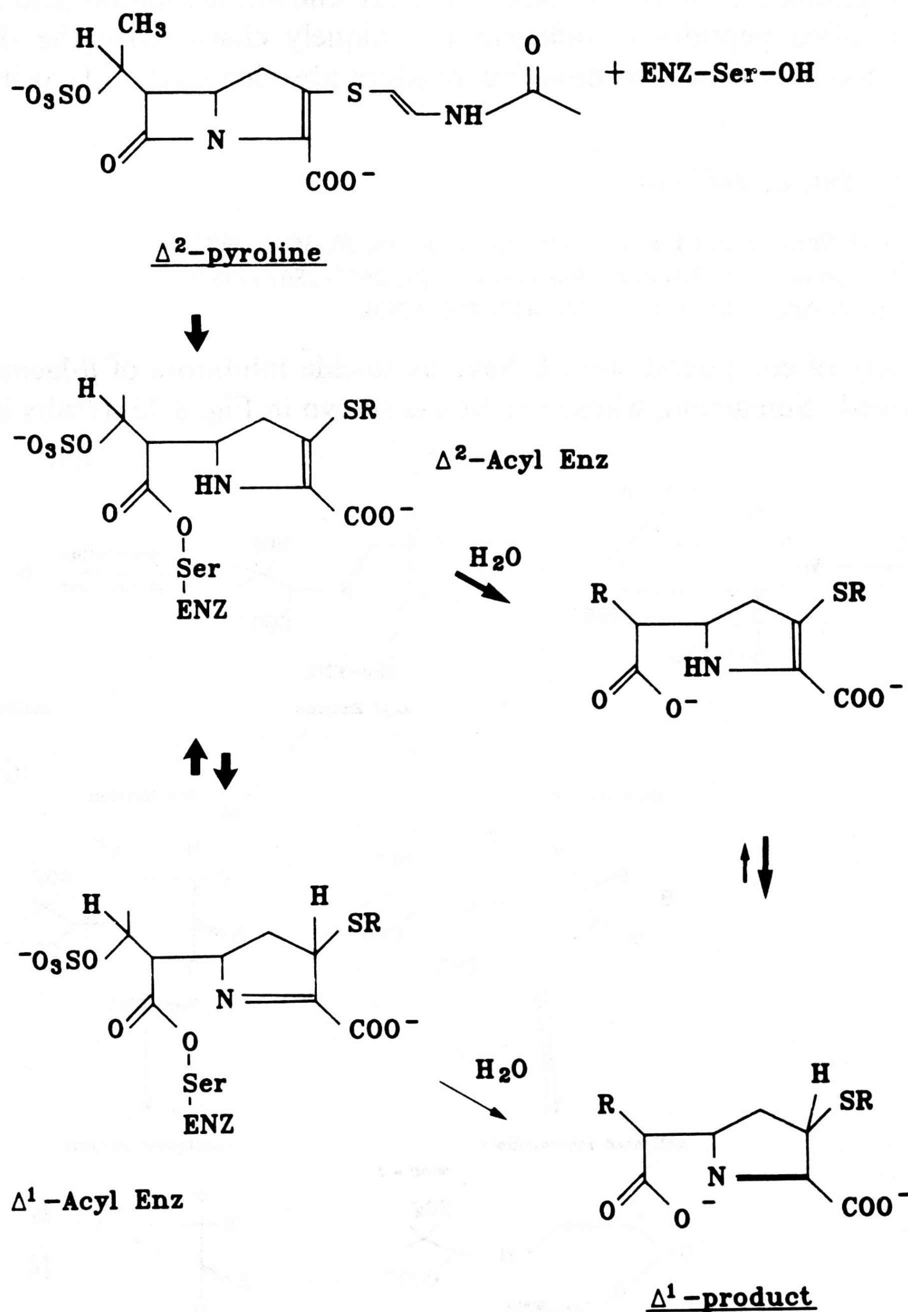


Figure 8-23 Δ^2-pyroline as a suicide substrate for β-lactamase.

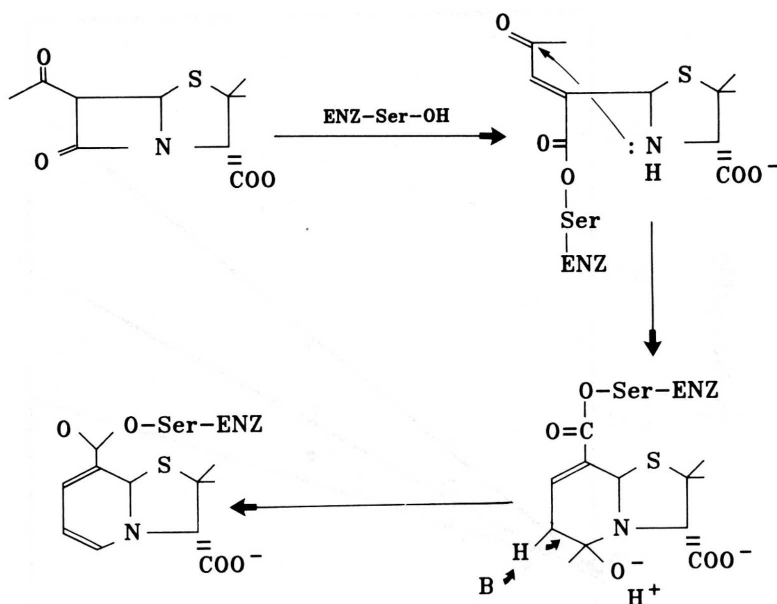


Figure 8-24 AcMPA as a suicide substrate of β-lactamase.

Finally, yet a third type of mechanism is observed, with acetylmethylene penicillanic acid (AcMPA) as substrate (Fig. 8-24). Acetylmethylene penicillanic acid leads to intramolecular cyclization of the acyl enzyme intermediate, which gives a product that does not hydrolyze, thus irreversibly inhibiting the enzyme.

Photo-Affinity Labeling of RNA Polymerase with 8-Azido-adenosine 5′-Triphosphate

Reference: A.-Y. Woody et al., *Biochemistry*, *23*, 2843–2848 (1984).

Initial rate kinetic studies (Fig. 8-25) show that 8-azido-ATP is a competitive inhibitor of the polymerase, with respect to ATP. When the kinetics with respect to UTP are examined, no inhibition by 8-azido-ATP is observed.

After irreversible labeling and analysis of the subunit distribution of the label, it is found that the β′ and σ subunits contained the majority of the label. The presence of ATP, but not UTP, causes a significant decrease in the amount of labeling, as shown in Table 8-3. These results indicate that the β′ and σ subunits of this multi-subunit enzyme contain the ATP binding sites, but that the β and α subunits are in close proximity. Labeling of the β and α subunits appears to be specific since it is reduced by the presence of ATP.

Although these examples have been carried to different extents, it is clear that affinity reagents have a tremendous potential to give information about the spatial location of binding sites, and their function. To get the maximum information from such studies requires a careful analysis of the location of modified residues in the primary sequence of the protein, together with documentation of the effects of specific

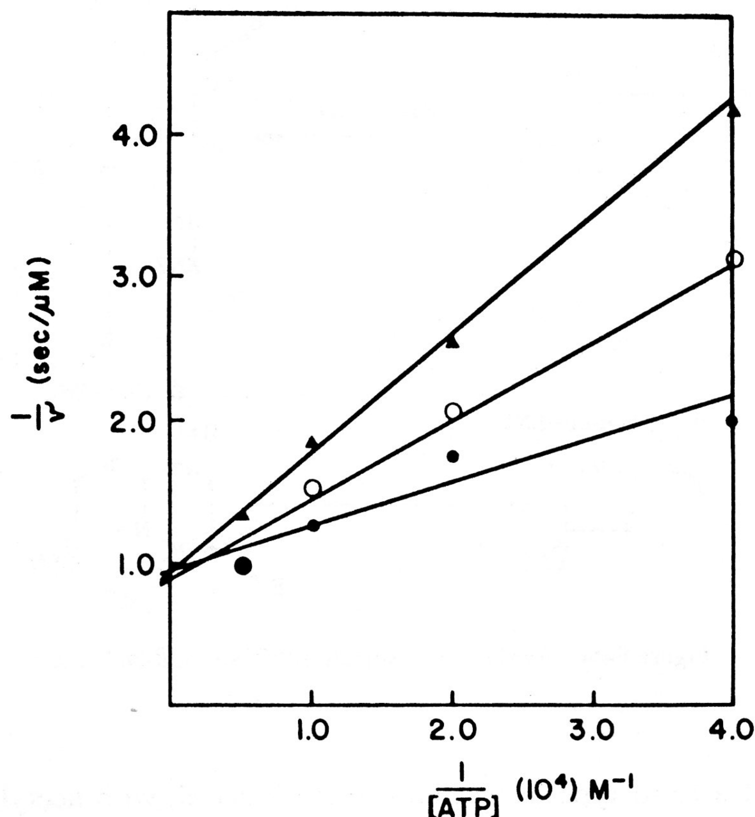


Figure 8-25 Competitive inhibition by 8-azido-ATP. Rate of RNA synthesis was followed in the absence of 8-azido-ATP (●) or in the presence of 25 M_μ (○) or 50 M_μ (▲) 8-azido-ATP. (Reprinted with permission from: A.-Y. M. Woody, C. R. Vader, R. W. Woody, and B. E. Haley, *Biochemistry, 23,* 2843–2848. Copyright 1984 American Chemical Society, Washington, D. C.)

modifications. The latter point is particularly necessary where conclusions regarding function of the site are to be drawn.

Affinity labeling has also found use in the introduction of specific, conformationally sensitive probes or fluorophores to be used in resonance energy transfer studies to estimate distances between points on a protein molecule.

TABLE 8-3 Effects of ATP and UTP on photoincorporation of 8-azido-ATP in subunits of RNA polymerase

Concentration (μM)		Percent incorporation in absence of ATP or UTP in each subunit			
ATP	UTP	β	β'	σ	α
0	0	100	100	100	100
35	0	75	40	66	57
150	0	20	11	15	11
0	100	100	120	110	
0	200	90	98	94	