Chemical modification of allosteric properties of enzymes

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Chemical modification is usually employed to study enzyme active sites. Valuable information can also be obtained, however, when this technique is used to probe allosteric sites. This approach is discussed in this article, and it is exemplified in chemical modification studies of the allosteric enzyme phosphofructokinase.

Key terms: allosteric enzymes; chemical modification; phosphofructokinase.

INTRODUCTION

As indicated in the article by Eyzaguirre (1996), chemical modification of enzymes can be monitored by changes in activity of the enzyme. Conclusions regarding the nature of the modification can be derived by a correlation of these activity changes with data on the stoichiometry of incorporation of the modifying reagent, which is usually facilitated by the presence of a chromophore or radiolabel on the reagent. The ideal situation is regarded as one in which there is a direct stoichiometric relationship between loss of enzyme activity and the incorporation of reagent into a single amino acid residue, either one involved in substrate binding or directly in the catalytic process. Examples of these are the classic studies of the modification of pancreatic proteases with diisopropylfluorophosphate or with chloromethylketone affinity reagents. Chemical modification of allosteric enzymes, on the other hand, provides a great deal more information while providing additional problems of monitoring the modification event and interpreting the data that are obtained. An example of problems of interpretation is illustrated by the contrast to a non-allosteric enzyme where site occupancy necessarily produces loss of activity, whereas occupancy of an allosteric site can produce several effects to be discussed below. Furthermore, instead of monitoring activity loss, one may be forced to measure loss of binding capacity to provide additional evidence for site occupancy by a modifying chemical reagent.

Despite the above mentioned difficulties, much information can be obtained by carrying out chemical modifications of allosteric enzymes. In addition to providing identity and sequence context for critical residues in a catalytic or allosteric site, chemical modification of allosteric enzymes may provide means of studying an enzyme that has been desensitized to allosteric control, establish that a regulatory site is topographically distinct from the catalytic site, or produce an enzyme that has been conformationally restricted or frozen into one of multiple conformation states available to the native enzyme. Once a procedure has been established for the production of an enzyme modified at a particular site, reagents bearing spin labels or fluorescent probes can be utilized to map inter-site distances within the molecule. In addition, hybrid molecules can be produced containing native and modified subunits to provide information concerning the transmission of conformational events and intersubunit communication. Prior to discussing the problems of interpreting the results of chemical modification of allosteric enzymes, it is appropriate to consider the general properties of this class of enzymes.

General properties of allosteric enzymes

Allosteric enzymes invariably consist of multiple subunits. Cooperativity is observed in the binding of substrate that produces
sigmoid substrate saturation curves in contrast to the hyperbolic response of non-allosteric enzymes. This is referred to as a homotropic effect and phenomenon is called homoallostery. The effect is interpreted to result from a conformational change of the subunits from a low affinity state to a high affinity state as a result of substrate binding. This can be viewed as conformational changes that result in the displacement of a pre-existing equilibrium between two affinity states (Monod-Wyman-Changeux model), as sequential, induced changes in affinity in adjacent subunits (Koshland-Nemethy-Fulmer model), or as a combination of these two extremes that may involve more than two states for the molecule.

Overlaid onto homoallostery is the action of heterotropic effects or the phenomenon of heteroallostery. Inhibitors or activators, negative or positive heterotropic effectors, respectively, bind to the enzyme at sites distinct from the substrate binding site but result in changes in affinity at the substrate binding site. If one considers the simple model of two conformational states, active and inactive, that are in equilibrium, then the action of inhibitors can be interpreted as binding of the inhibitor preferentially to the inactive state, which consequently displaces the equilibrium toward that state. Conversely, activators would bind with higher affinity to the active conformation and displace the equilibrium in that direction. In the sequential model, the effectors induce upon binding the appropriate conformation to change affinity for the substrate. The sequential model allows for negative cooperativity; that is, the situation where binding to the first subunit of a polymeric enzyme promotes a conformational change in adjacent subunits that lead to a decrease in affinity. An extreme case of negative cooperativity is half-of-the-sites reactivity. In these unusual instances, binding of a ligand to one subunit in a dimer or two subunits of a tetramer effectively prevents binding of the ligand to the other subunits.

Consequences of chemical modification of an allosteric enzyme

As indicated, there are a number of potential consequences of chemical modification of allosteric enzymes. Activity losses can occur as a result of a specific modification of a critical residue or residues in the substrate binding pocket or of those residues intimately involved in the catalytic process. Generalized loss of activity can also occur as a result of modifications that produce discrete conformational changes that disrupt the active-site or more generalized disruption of overall secondary, tertiary, and/or quaternary structure of the protein. Of greater interest here are those modifications that result in alterations of regulatory properties. Such modifications may be grouped into three general types.

First, the modification can be a productive interaction with the regulatory site in which the enzyme behaves as it does when the site is occupied by the natural dissociable ligand. In this instance the enzyme is frozen irreversibly in a particular conformation, with higher or lower activity, depending upon the natural ligand's action. Second, the modification can react with residue(s) within the allosteric site to prevent interaction of the natural ligand while not yielding a productive conformational change on its own. This would desensitize the enzyme to the ligand that binds to that particular site. It should be noted that partial effects can be produced. Because a given binding pocket contains a number of residues important to binding, destruction of any one may simply decrease, but not abolish affinity for the regulatory ligand. Third, there are modifications that prevent or modify the allosteric transitions or conformational changes that occur as a result of the binding of regulatory ligands. In other words, binding of regulatory ligand occurs, but no regulation results because the necessary conformational change does not occur. The most readily observed of this last type are those that result in dissociation of multi subunit enzymes, thus preventing inter-subunit transmission of conformational events associated with substrate or regulatory ligand interaction. One can also conceive of modifications that block a residue critical to intra- or inter-subunit communication that would result in some degree of loss in the transmission of conformational events.
In this article, the aforementioned consequences of chemical modification of allosteric enzymes will be discussed with concrete examples. In addition, the methodology for studying those changes in structure and function that occur upon chemical modification will be presented. Although extensive studies of many allosteric enzymes have been carried out over the last 30 years, most of the discussion here will relate to work done by the author and others with an enzyme of exquisite allosteric complexity: mammalian phosphofructo-1-kinase. The following section will briefly describe the properties of this enzyme.

PHOSPHOFRUCTO-1-KINASE: A MODEL OF ALLOSTERICISM

Mammalian phosphofructokinases, which carries out the phosphorylation of fructose 6-P with ATP to produce fructose 1,6-P₂ and ADP, is tetrameric with subunit sizes in the range of 80 to 84 kDa, depending upon the animal source and the isozyme under consideration. The enzyme is under regulatory control and is thought to be the principal pacemaker in glycolysis. The most important regulatory properties are typified in the three panels of Figure 1. The enzyme is inhibited by high concentrations of one of the substrates, ATP (Fig 1A), and the inhibition is reversed in the presence of the activators AMP (or ADP) and fructose 2,6-P₂ (or fructose 1,6-P₂). The enzyme displays a striking sigmoid response toward the concentration of the other substrate, fructose 6-P (Fig 1B), and the activators produce a decrease in fructose 6-P₀₅ and a decrease in the interaction coefficient (slope in a Hill-type plot). The action of the other major inhibitor citrate is synergistic with ATP inhibition (Fig 1C), i.e., a given concentration of citrate will inhibit at a low non-inhibitory concentration of ATP, and that sensitivity to inhibition is enhanced at higher but still non-inhibitory concentrations of ATP. Equilibrium binding studies have shown that each subunit has six organic ligand binding sites: MgATP and fructose 6-P (the catalytic site), MgATP and citrate at inhibitory sites, and sites for each of the activators, AMP and fructose 2,6-P₂. Inorganic phosphate and ammonium ion are also activators, but the number of sites of

![Fig 1](https://example.com/image.png)

**Fig 1.** Stylized representation of the allosteric properties of phosphofructokinase. Panel A: ATP inhibition curve at pH 7.2 plus and minus 20 • M AMP or 0.5 • M fructose 2,6-P₂. Panel B: Fructose 6-P saturation curve plus and minus AMP or fructose 2,6-P₂. Panel C: Citrate inhibition at two non-inhibitory concentrations of ATP.
interaction has not been determined. These allosteric phenomena have been associated with conformational changes in the protein as monitored by thiol reactivity, intrinsic fluorescence changes, extrinsic fluorescent probes, and sensitivity to proteolysis. For a more extensive discussion of these and other characteristics of mammalian phosphofructokinase, the reader is recommended to the review by Kemp and Foe (1983).

**METHODS TO STUDY THE CONSEQUENCES OF MODIFICATION OF ALLOSTERIC ENZYMES**

**Kinetic studies**

As noted above, chemical modifications of non-allosteric enzymes basically produce only one type of result, a change, usually a decrease, in the activity of the enzyme. Activity losses may reflect either a loss in catalytic efficiency or an increase in \( K_m \) for substrate. For the most part, these consequences will be revealed in initial velocity kinetic studies. Other more complicated scenarios can be imagined with two or three substrate reactions wherein mechanistic alterations occur such as changes in the distribution among reaction paths in a random mechanism. Such changes are also to be expected if an allosteric enzyme is modified at or near its catalytic site. The interpretation here is much more complex, however, because of cooperativity in the binding of substrate by interacting subunits. One must also distinguish between a modification at the catalytic site and one at an allosteric site wherein the kinetic consequences may be nearly identical.

The first problem of interpretation is one of the appropriate graphical representation of initial velocity data. The Lineweaver-Burke plot or any of the straight-line equation rearrangements of the Michaelis-Menten equation are of no use in the interpretation of data that show cooperativity. While a number of non-linear representations of the data have utility, including the basic \( V \) vs \( S \) plot, the most widely used graphical representation is the Hill plot for the binding of oxygen to hemoglobin, as adapted to kinetic data. Usually called the ‘Hill-type’ plot, it plots the log \( V/(V_{max} - V) \) vs log \( S \), and gives useful straight-line data between velocity values of 10 to 90% of \( V_{max} \). The slope of the Hill plot yields a number usually referred to as the interaction coefficient' (or simply \( n \)), which is a measure of the degree of cooperativity in the binding of substrate or ligands. An enzyme that obeys Michaelis-Menten kinetics would give an interaction coefficient of one. Positive cooperativity gives values greater than one, negative cooperativity yields values less than one. An example of the use of the Hill-type plot will be presented later in this article (see Fig 6).

**Ligand binding studies**

Kinetic studies of allosteric enzymes often cannot distinguish between a modification directly at a substrate site vs one that occurs at an allosteric site and influences the catalytic site. Similarly, one cannot distinguish kinetically from the loss of interaction at a regulatory site vs a disruption in conformational signal, such as that produced by dissociation of subunits. Ligand binding studies provide information about the site that has undergone modification. One compares native and modified enzyme in terms of both the maximum number of moles of ligand bound as well as the affinity of the enzyme for the ligand. This requires that binding be performed at varying substrate concentration and the data plotted appropriately. If the binding adheres to a hyperbolic isotherm, then any of the familiar graphical techniques of enzyme kinetics can be employed, substituting moles bound for the velocity term. The most familiar graphical representations is that of the Scatchard-type plot, wherein \( C \), the number of moles bound per mole of protomer, divided by \( L \), the concentration of free ligand, is plotted against \( C \). The slope provides the dissociation constant, while the \( X \) intercept gives the binding unit. The Scatchard plot often gives interpretable data for negative cooperativity, half-the-sites reactivity, or multiple binding sites. If cooperative binding is observed, then a Hill-type plot can be used, substituting moles bound for the velocity terms.

One of the earliest binding methods employed was that of equilibrium dialysis.
(e.g., Changeux et al., 1968). Ligand and protein are mixed and placed in a dialysis bag suspended in an appropriate buffer. At equilibrium, the concentration of ligand outside the bag represents free ligand, and that inside is bound plus free. The method has the disadvantage of being slow because of the time required to achieve equilibrium. An interesting variation on the theme of dialysis was the approach of Colowick and Womack (1969) who devised an analysis of the free ligand concentration based upon the rate of flow across a semipermeable membrane into a chamber that was to be continuously flushed into a fraction collector. In such a system a single protein sample can be used for an entire binding curve, and a whole series of data points can be collected within an hour. The technique works most effectively with radiolabeled ligands available at a fairly high specific activity and when the affinity is high. A precise but time consuming variation on equilibrium dialysis is the method of Hummel and Dreyer (1962). The protein is passed through a column of Sephadex G25 or G50 previously equilibrated with ligand, usually radiolabeled. The protein will continue to bind more ligand until it comes into equilibrium with the concentration of ligand on the column. Fractions are collected, and the protein peak will be the sum of free and bound ligand, while the plateaus before and after the peak represent free ligand. The shape of the elution profile provides clues to non-equilibrium or to other artifacts such as instability of the protein or the ligand. While the method is tedious, analysis time can be reduced by using an FPLC system equipped with a Fast Flow Desalting Column (Pharmacia).

Another technique that uses the exclusion properties of Sephadex to separate free ligand is a method described by Kitajima and Uyeda (1983). Small columns of Sephadex are placed in a centrifuge tube and sedimented at low speeds to facilitate the separation.

Yet another method is the membrane filtration technique which has been described by Paulus (1969). Free ligand concentration is determined from a filtrate after pushing a portion of ligand-protein mixture through an ultrafilter. This is a fairly widely used technique, although problems can occur if either the ligand or the protein interact with the filter.

Several of these techniques find their antecedent in the sedimentation technique that was apparently first utilized by Hayes and Velick (1954). Binding of NAD to a dehydrogenase was demonstrated by centrifuging protein plus NAD at high speeds to concentrate the protein at the bottom of the tube. Samples in the protein-free supernatant and the protein layer could be analyzed for NAD to determine free and bound plus free, respectively. While this technique was time-consuming as first described, the more recent utilization of ultracentrifuges that handle very large numbers of samples makes the technique more attractive. The problem with this and any technique that leads to concentration of the protein is that misleading results will be obtained if the protein shows concentration-dependent changes in ligand-binding behavior. Such behavior is not unusual among allosteric enzymes. Virtually every technique has the problem that one employs protein concentrations far greater than that used in most kinetic assays. At high protein concentrations, the behavior of many enzymes differs from that seen at very low concentration as a result of concentration dependent association behavior.

An entirely different class of binding techniques involves the use of optical methods. Changes in fluorescence of the ligand upon binding can be readily quantified and saturation curves developed. This method has been widely used to study the binding of NAD and its analogs to dehydrogenases. Fluorescent derivatives of ligands that do not normally display fluorescence have been used, such as the etheno-derivatives of adenine nucleotides (e.g., Liou & Anderson 1978). Still another binding technique follows the quenching of intrinsic fluorescence of tryptophan or tyrosine residues in the protein. One note of caution here, however, is that the fluorescence change can result from a conformational event associated with binding. Because a modification could disrupt the sequence of events that produces that conformational change without greatly affecting the binding event itself, a failure to
elicit a change in intrinsic fluorescence typical of the native enzyme upon exposure to ligand does not necessarily imply loss of binding. This caution should be exercised as well with other methods that are probably based upon conformational events. To assume that changes in reactivity of surface residues or changes in resistance to heat inactivation or to limited proteolysis is a result in changes in binding may well be inappropriate. The change in reactivity or sensitivity may be due to a failure to produce a conformational change and may be independent of the binding event itself.

Finally, an excellent approach to binding studies that has not been used extensively with allosteric enzymes is microcalorimetry. Within a relatively short period of time one can determine binding constants and stoichiometry of ligand interaction as well as the heat and entropy of binding. It may be possible in some instances to sort energy changes associated with binding and those associated with accompanying conformational events.

At present, the required instrumentation is expensive and not available to all laboratories.

Conformational studies

It is possible that binding may not elicit the conformational change required to change activity. The classic example of this are the studies of aspartic transcarbamylase where binding of regulatory ligand is not decreased despite the loss of an allosteric effect by dissociation of the catalytic and regulatory subunits. It is possible that binding may not elicit a change in intrinsic fluorescence typical of the native enzyme upon exposure to ligand does not necessarily imply loss of binding. This caution should be exercised as well with other methods that are probably based upon conformational events. To assume that changes in reactivity of surface residues or changes in resistance to heat inactivation or to limited proteolysis is a result in changes in binding may well be inappropriate. The change in reactivity or sensitivity may be due to a failure to produce a conformational change and may be independent of the binding event itself.

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It is possible that binding may not elicit the conformational change required to change activity. The classic example of this are the studies of aspartic transcarbamylase where binding of regulatory ligand is not decreased despite the loss of an allosteric effect by dissociation of the catalytic and regulatory subunits (Changeux et al., 1968). Furthermore, occupancy of the same site by two different ligands does not necessarily produce the same conformational or kinetic result (Kemp & Foe, 1983). Obviously, a study of the consequences of chemical modification should include evidence for, or excluding, perturbation in the normal pattern of conformational change associated with the transition between activated and inhibited state. Changes in protein conformation can be determined by a variety of optical techniques that have been employed for many years to study ordered structures in proteins. These include optical rotatory dispersion, circular dichroism, and intrinsic fluorescence measurements. Changes in polymeric state can be monitored by analytical ultracentrifugation, by analysis of Stokes radius by gel exclusion chromatography, or by sucrose gradient sedimentation. Different reactivity of functional residues on the protein surface also provides evidence for different protein conformations under varying conditions. Trace labeling of amino groups with acetic anhydride or other highly reactive acylating agents coupled with HPLC mapping of tryptic digests can provide evidence for different conformational states. The author's laboratory has used extensively the reactivity of thiol groups as a conformational monitor. Thiol groups are attractive because they can be readily modified by a great number of reagents such as a variety of disulfide interchange reagents (e.g., Ellman's reagent) and a number of alkyl halides. Still another indicator of conformational events is differential reactivity toward proteolytic enzymes. In any of these techniques based on differential reactivity, there remains one uncertainty: a loss in reactivity of a protein side-chain or the diminished susceptibility of a particular peptide bond to a protease can result either from a direct shielding of the residue or protease recognition site by a ligand or from a conformational event associated with ligand binding. Other evidence based on results from other ligands or on other types of conformational analysis would be required to distinguish between these possibilities.

STUDIES ON THE MODIFICATION OF PHOSPHOFRUCTOKINASE

Modification studies of phosphofructokinase have been extensive, leading to all of the potential consequences mentioned previously, that is, productive modifications to freeze the enzyme in activated or inhibited conformations, modifications that prevent ligand induced action by blocking a regulatory site, and modifications that interfere with conformational transitions. An example of the first type is shown in studies by Mansour and Coleman (1978) who employed the adenine nucleotide analog, F-sulfonylbenzoyl-adenosine. These studies originally performed on sheep heart muscle phosphofructokinase
produced an activated enzyme largely desensitized to ATP inhibition. We have repeated this modification on the purified isozymes of phosphofructokinase from rabbit skeletal muscle and rabbit liver (Valaitis et al., 1988). Despite considerable similarity in the properties of these two isozymes, the chemical modification by F-sulfonylbenzoyl-adenosine proceeded quite differently with the liver enzyme providing results that are instructive with regard to the phenomenon of half-of-the-sites reactivity. Figure 2 describes the time course of modification of the muscle and liver isozymes by the adenosine derivative. The muscle enzyme incorporates four moles of modifying reagent per tetramer with a time course that indicated identical reactivity of all subunits. Although the liver enzyme (lower line of Fig 2) also is a tetramer of identical subunits, it was modified to the extent of about 0.5 moles per monomer. Phenomena of this type are thought to be associated with conformational changes that prevent further incorporation of reagent after two sites of the tetramer are occupied. In the case of the muscle enzyme, the reaction of the adenosine derivative with the enzyme brought about a productive interaction similar to that caused by the natural dissociable activator. The modified muscle enzyme appeared to be frozen in the active state as indicated by the failure of ATP to inhibit the enzyme (Fig 3, upper part). Because the liver enzyme was only partially modified by the reagent, it remained inhibitable by high ATP, although the inhibition was less than that observed with native liver enzyme (Fig 3, lower part). On the other hand, the AMP binding site of the muscle enzyme was totally blocked as shown by the failure of the modified enzyme to bind cyclic AMP in a Hummel-Dreyer binding column (Valaitis et al., 1988).

A covalent modification at an inhibitory site is typified by the modification of phosphofructokinase by pyridoxal phosphate (Colombo & Kemp, 1976). In these experiments, pyridoxal phosphate was reduced onto the enzyme with NaBH₄, and several lines of evidence indicated that one mole of pyridoxal phosphate was incorporated almost exclusively in the citrate inhibitory site of the enzyme. The modified enzyme had decreased activity and exquisite sensitivity to ATP inhibition (Fig 4A). Binding studies with labeled ATP showed that the enzyme had enhanced affinity for ATP as would be expected by the synergistic inhibition displayed by ATP and citrate (Fig 1C). On the other hand, citrate binding was blocked as shown by the Scatchard plot in Figure 4B. In this instance the enzyme had been modified by the incorporation of about 0.85 moles of pyridoxal phosphate. The data show that what binding remained was undoubtedly due to the unmodified fraction which bound citrate with an affinity equal to that of native enzyme. If the data had shown that the residual binding had identical maximum binding but lower affinity, one would conclude that the modification was not at the citrate site but only influenced the citrate site. This demonstrates the importance of relatively complete binding studies in interpreting such data. The modification by pyridoxal phosphate and sodium borohydride has thus frozen the enzyme in an inhibited conformation by a covalent linkage to a lysine residue in the citrate pocket. Isolation and sequencing of a labeled tryptic peptide identified the lysine as residue 540 (Kemp et al., 1987).

An example of a modification that destroys a binding site can be seen in the
Fig 3. ATP inhibition of native and modified phosphofructokinases. Upper part: native (o) and modified (△) muscle phosphofructokinase. Lower part: native (o) and modified (△) liver phosphofructokinase. Data taken from Valaitis et al. (1988).

experiments of Setlow and Mansour (1970) who modified sheep heart phosphofructokinase with diethylpyrocarbonate. Incorporation of close to four moles of reagent per mole of protomer virtually abolished ATP inhibition. Setlow and Mansour concluded that one or more histidines at the ATP inhibition site were modified. We repeated these experiments with rabbit muscle phosphofructokinase and obtained nearly identical kinetic results (Fig 5A). Figure 5B demonstrates the loss of conformational change to the inhibited conformer following modifications as indicated by thiol reactivity. One thiol group of phosphofructokinase, identified as Cys-88 (Latshaw et al., 1987), is extremely reactive; and this reaction with dithionitro(bis)benzoic acid (DTNB) is inhibited in the presence of ATP. Previous studies have shown that this is a result of ATP binding to an inhibitory site that produces a conformational change (Kemp, 1969). In Figure 5B, it can be seen that the ethoxyformylated enzyme retains the highly reactive thiol, but its reaction is not inhibited by ATP. These data provide confirmation at the level of allosteric transitions that the interaction of ATP at the inhibitory site has been abolished.

The final potential consequence of chemical modification is typified in a kinetic analysis of phosphofructokinase before and after the modification of the highly reactive thiol group described above. The result is activation of the enzyme at low concentrations of fructose-6-P and sharp reduction in cooperative kinetics (Kemp, 1969). This might be interpreted as a blockade of the ATP inhibitory site. The results are not nearly so simple, as indicated by the Hill-type plot shown in Figure 6. This figure shows the two effects of ATP on the kinetics of the unmodified enzyme (open symbols): higher ATP increases the concentration of fructose-6-P necessary to achieve half-saturation and it increases the interaction coefficient. The modified enzyme retains the effect of ATP on half-saturation; thus ATP must continue to interact with the enzyme although the full expression of cooperative behavior has been lost. The interpretation is that binding is not affected but the conformational change associated with the binding is partially blocked. This is consistent with the previously mentioned data that this thiol group is an indicator of the active-inactive transition and must occupy a site that undergoes change during conformational events. One can imagine that a bulky reagent attached to this thiol may inhibit the conformational change. To complete this study and verify such conclusions, ligand binding of the native and thiol-modified enzyme should be performed.

Chemical modification versus site-directed mutagenesis

As more and more allosteric enzymes are cloned and expressed, there is no doubt that
site-directed mutagenesis will supplant much of the use of chemical modification as a tool to study the mechanism of allosteric interactions and the role of specific amino acid residues in those mechanisms. Site-directed mutagenesis eliminates the problem of uncertainty with regard to the extent of modification because the modification is always 100%. This allows an analysis of residual properties that can provide much more information than a simple yes or no that one gets with chemical modification. However, one not only needs a cloned and expressed enzyme to carry out efficient mutagenesis, one needs to have some clue as to what residues may be involved, as determined from X-ray structure studies or from information about homologous proteins. One the other hand, chemical modification can proceed without information.
concerning structure. In fact, it is chemical modification data that can provide the important clues to structures of significance that can subsequently be subjected to detailed analysis by mutagenesis.

REFERENCES


