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Affinity labels as probes for the study of the nucleotide binding site of enzymes

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Affinity labeling has proved to be a very useful tool for searching important amino acid residues located in active or allosteric sites of enzymes. In this article, the general principles and specific examples of the use of affinity labeling are discussed.

Key terms: *affinity labeling; fluorosulfonylbenzoyl analogs; nucleoside polyphosphopyridoxals; other nucleotide derivatives; periodate-oxidized nucleotides*

GENERAL CONCEPTS

Enzymes are highly specific biological catalysts. This is primarily due to the capacity of the enzyme active site to form specific complexes with its substrates to promote chemical reactivity.

Numerous studies have been performed on the active site of enzymes in order to understand their mechanism of action. In regulatory enzymes the structure of sites that bind allosteric effectors is also important. One method of obtaining information about active or allosteric sites of enzymes is through chemical modification. This may be accomplished by using specific reagents directed to distinct chemical groups in the protein, or by means of affinity labels. The goal is to produce a change in some property of the enzyme that can be correlated with the functional role of specific amino acid residues. The specific chemical modification ideally results in the quantitative modification of a functional group belonging to a unique amino acid residue without affecting other functional groups or the conformation of the enzyme molecule. The limitations of the chemical modification approach in active-site studies is discussed in the article by Eyzaguirre.

Affinity labels have proved to be powerful tools in the study of the relationship between structure and function of proteins. These reagents, also called site-specific reagents, are characterized by being structurally similar to known substrates, allosteric effector or other ligands that bind to proteins, and therefore show affinity towards these ligand binding sites. These compounds are being extensively studied, and many affinity reagents have been designed for a number of proteins. Several reviews, some very recent ones, have appeared on the subject (5, 6, 7, 38, 40) including a special volume of Methods in Enzymology (20).

Affinity labels differ from reversible inhibitors in that they possess reactive groups capable of forming covalent bonds with amino acid side chains. The reactive groups are usually alkylating or arylating agents. A very important class of affinity reagents are the photoaffinity labels, which have a photolabile group in their structure. These agents are activated by irradiation, forming *in situ* a very reactive functional group (usually a carbene or nitrene) which is capable of interacting with the neighboring chemical residues. In this way it is possible to modify hydrophobic or polar residues which are normally of low reactivity (16).

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Photoaffinity reagents are further discussed by Schäfer in this work.

Affinity labeling involves at least two steps: the site-specific binding of the reagent and the subsequent modification of an amino acid residue through the formation of a covalent bond. Affinity labeling must show saturation kinetics (34, 51). It can be demonstrated that the kinetic process corresponds to the following scheme:

$$P + A \underset{k_2}{\overset{k_1}{\Leftrightarrow}} P^*A \xrightarrow{k_3} P\text{-}A \qquad (1)$$

$$Ka = \frac{[P][A]}{[P^*A]} = \frac{k_2}{k_1} \quad k_{obs} = \frac{k_3[A]}{[A] + K_a} \qquad (2)$$

where P is the protein, A the affinity label, P^*A the non-covalent (Michaelis-type) complex, P-A the covalent product of the reaction and K_a the dissociation constant of the non-covalent complex. The designation of K_a as k_2/k_1 depends on an assumption of quasi-equilibrium around P^*A. This is probably a valid assumption except for very rapid modification reactions where the apparent second-order rate constant under conditions of low [A] is greater than *ca.* $10^6\ M^{-1}\ s^{-1}$ (3).

The pseudo-first-order inactivation rate constant (k_{obs}) is obtained upon conditions where the modifying reagent is present in great excess in relation to the protein. According to equation 2, the pseudo-first-order inactivation rate constant presents saturation kinetics at high reagent concentration; this is one of the requirements that a reagent must satisfy in order to be considered as affinity label. By the very nature of the phenomenon under study, it is expected that a substrate, competitive inhibitor or ligand will protect against modification and inactivation. In addition, the reaction must be directed exclusively to the functional subunit of the enzyme.

REAGENTS COMMONLY USED TO MODIFY PURINE-NUCLEOTIDE BINDING SITES IN PROTEINS

Purine nucleotides are very important in metabolism. They are involved in reactions of kinases, carboxykinases and ATPases among others, and they function as allosteric effectors of many enzymes. Moreover, as part of NAD^+ or $NADP^+$, they participate in reactions catalyzed by dehydrogenases. This review will focus on the labeling of nucleotide sites in enzymes, with emphasis in the discussion of oxidized nucleotides.

1. *Periodate-oxidized nucleotides*

This group of compounds presents several advantages such as easy of preparation and solubility in aqueous solvents similar to that of the non-modified nucleotides. They can also be easily prepared as radioactive labels. In general, periodate-oxidized nucleotides behave as affinity labels of enzymes, although a few cases of non-specific reactions are reported (33).

The oxidation of ribonucleotides by periodate results in cleavage between the 2'-3' carbons of the ribose moiety to yield the corresponding dialdehyde derivative. This destruction of the ribose ring could be important in the binding of the nucleotide to certain enzymes. Aldehydes are capable of reacting with primary amines such as the ε-amino group of lysine or the terminal α-amino group of proteins. Periodate-oxidized ATP has been extensively characterized by NMR spectroscopy (31).

One of the earlier reports on the use of these compounds was the study of the effect of 2',3' dialdehyde ATP (oATP) on sheep liver mitochondrial pyruvate carboxylase (14). It was found that the Mg-oATP^{-2} complex behaved as a competitive reversible inhibitor with respect to Mg-ATP^{-2}. When $NaBH_4$ was added to the reaction mixture, Mg-oATP^{-2} became covalently bound to the enzyme, producing irreversible inactivation. These results agreed with the formation of a Schiff's base, which is reduced by $NaBH_4$. Mg-ATP^{-2} protected the enzyme against chemical modification, suggesting that oATP was binding to the nucleotide site. Total loss of activity was observed when one mole of Mg-oATP was incorporated per mole of enzyme subunit. The modified amino acid was identified as lysine following enzymatic digestion of the Mg[^{14}C]oATP^{-2}-labeled enzyme and chromatography using [^{3}H]Lys-oATP as standard.

The use of periodate-oxidized $NADP^+$ ($oNADP^+$) was introduced by Dallocchio *et al* (10). They showed that oNADP+ could be bound to *Candida utilis* 6-phosphogluconate dehydrogenase producing reversible inactivation of the enzyme. The inactivation was made irreversible by $NaBH_4$ reduction. These investigators chemically synthesized the two expected products from the reaction between the aldehyde groups of carbon 2' or 3' of the modified ribose and a lysine residue of the enzyme and its subsequent reduction with $NaBH_4$ and hydrolysis. Chromatographic analysis showed that the products derived from the reaction between the enzyme and oNADP behaved identically to the chemically synthesized standards. These derivatives have been separated and identified by amino acid analysis (24).

Another communication reporting the formation of a Schiff's base between enzyme and oxidized nucleotides (25), shows that oATP behaves as a good affinity label for the latent ATPase from *Mycobacterium phlei*. This reagent produced the progressive inactivation of latent and unmasked activities of the ATPase with non-linear dependence between the pseudo-first-order inactivation rate constant and oATP concentration. These results indicate the formation of a reversible complex prior to the covalent modification. The substrate ATP protected the enzyme against inactivation. At 100% inactivation, one mole of ATP was incorporated per mole of enzyme.

The effect of oATP on partially purified adenylate cyclase from bovine brain has also been studied (49). The analog behaved as a competitive inhibitor of the enzyme. The simultaneous treatment with oATP and $NaCNBH_3$ produced irreversible inactivation of the enzyme with pseudo-first-order kinetics. A hyperbolic relationship between inactivation constants and oATP concentration was found. ATP and Tris protected the enzyme against inactivation. These data suggest the formation of a Schiff's base between the enzyme and oATP, although this could not be verified because of lack of pure enzyme.

Chemical bonds different from Schiff's bases have also been detected in the reaction between an enzyme and a periodate-oxidized nucleotide. An example is found in rabbit muscle phosphofructokinase (PFK) treated with dialdehyde-ATP (17). oATP produced enzyme inactivation which was not reversed by dialysis. A 99% inactivation was achieved with the simultaneous incorporation of 3-4 moles of the analog per subunit. ADP and ATP partially protected the enzyme from inactivation. Amino acid analysis of the modified enzyme did not show any difference from the untreated enzyme, whether or not $NaBH_4$ was added. These results suggest that the product of the reaction between oATP and PFK does not correspond to a Schiff's base. Furthermore, the modified form of PFK was stable in 0.01 N HCl, which is inconsistent with the lability expected for a Schiff's base. These authors proposed that the lysine residues of PFK react with oATP forming morpholine-type adducts, a result not previously observed between the reaction of lysine residues of proteins with aldehyde reagents.

The 2',3'-dialdehyde derivative of ATP produced the inactivation of skeletal muscle phosphorylase kinase (22). The reaction was pseudo-first-order with saturation kinetics and the natural substrate, ATP, protected against inactivation. Furthermore, oATP could be used as a substrate to phosphorylate phosphorylase b, supporting the suggestion that oATP binds to the active site of the enzyme. The oATP-inactivated enzyme could not be reactivated by dilution with buffer or by dialysis. The treatment of the enzyme-oATP complex with $NaBH_4$ or $NaCNBH_3$ did not affect the degree of inactivation. These results suggest that oATP binds strongly to the enzyme, but the inactivation product is not a Schiff's base.

The 2',3'-dialdehyde derivative of 8-azido adenosine 5'-triphosphate (8-N_3oATP) has been used on phosphorylase kinase (23). This compound possesses the dialdehyde groups of oATP and is also a photoaffinity reagent containing an azido group. The enzyme became inactivated in the presence of the analog in the absence of photolysis, and showed similar behavior to the previously reported results with oATP (22). The results showed a similar K_i for 8-N_3oATP and the ability to serve as substrate. Upon irradiation the analog was incorporated to the enzyme,

preferentially labeling the ß subunit, similar to the effect observed with 8-azido adenosine 5'-triphosphate. This latter compound prevented the incorporation of 8-N_3oATP into the enzyme, suggesting that both azido analogs compete for the same binding site.

Pig heart NAD-dependent isocitrate dehydrogenase was modified by oADP (24). The enzyme was inactivated with biphasic pseudo-first-order kinetics. For both phases a non-linear relationship between rate constant and reagent concentration was found. ADP and isocitrate in the presence of Mn^{+2} produced a significant reduction in the rate of inactivation, and ADP seemed to compete with the analog for the same nucleotide binding site. The incorporation of approximately one mole of [^{14}C]oADP per mole average subunit produced the complete inactivation of the enzyme. The protease-digested [^{14}C]oADP-inactivated enzyme did not form the lysine derivatives expected for a Schiff's base between oADP and an amino group of the enzyme; all the radioactivity was associated with neutral amino acids even though a negatively charged product was expected. Moreover, the reduction of the oADP-labeled enzyme with [^{3}H]$NaBH_4$ did not incorporate ^{3}H to the enzyme. These results provide evidence against a Schiff's base product for the inactivation of isocitrate dehydrogenase by oADP, and suggest a reaction mechanism in which both aldehydes groups become unavailable for reduction.

In a previous study on the inactivation of mitochondrial adenosine triphosphatase by oATP (30) it was proposed that after the binding of oATP to the enzyme an elimination reaction occurs, which liberates the triphosphate group and forms a very stable conjugate Schiff's base. Therefore, isocitrate dehydrogenase was modified with both [^{14}C]oADP and [^{32}P]oADP under standard conditions to test for ß elimination of the pyrophosphate group from oADP bound to the enzyme. The authors found a dramatic difference between binding of [^{14}C]oADP and [^{32}P]oADP per mole average subunit, [^{32}P]oADP being bound in very small amounts. They also showed that the release of [^{32}P]-pyrophosphate occurred after oADP binding to the enzyme. The elimination reaction may lead to the formation of a stable conjugate Schiff's base which may not require reduction by $NaBH_4$ to stabilize the analog on the enzyme (32). This type of reaction should have allowed for incorporation of ^{3}H into the unreacted 2' aldehyde group, however, when the oADP-labeled isocitrate dehydrogenase was reduced with [^{3}H]$NaBH_4$. A possible reaction product involving both aldehyde groups of oADP is a dihydroxymorpholine derivative, as suggested for phosphofructokinase inactivated by oATP (17). This derivative is relatively stable in dilute acid (0.01 N HCl), but the hydrolysis with 6 N HCl regenerates the unmodified lysines. The reaction product between oADP and isocitrate dehydrogenase most consistent with the available data is a 4',5'-didehydro-2',3'-dihydroxymorpholine derivative of lysine. In spite of the relative stability of the product, attempts to separate the proposed derivative have been unsuccessful.

Rabbit muscle pyruvate kinase has been modified with oADP (18). The enzyme was inactivated in a pseudo-first-order reaction with saturation kinetics. These results indicate that the enzyme formed a reversible complex prior to covalent modification. ADP and ATP, especially in the presence of Mg^{+2}, protected the enzyme against inactivation. oADP was not a substrate, but acted as a competitive inhibitor with respect to ADP. The reaction between oADP and pyruvate kinase seems to be very specific. Figure 1 shows that in the presence of 25 mM MgATP the incorporation of [^{14}C]oADP was very low; in the absence of the protective compounds the enzyme rapidly incorporated about 1 molecule of [^{14}C]oADP per subunit. The inactivation reaction was neither reversed by removal of excess of oADP by exhaustive dialysis or gel filtration, nor was any reactivation observed after addition of Tris or lysine to the inactivation mixture. The addition of $NaBH_4$ was not necessary to stabilize the linkage formed. In fact, the addition of [^{3}H]$NaBH_4$ to the oADP-modified pyruvate kinase did not incorporate ^{3}H to the enzyme. The mechanism for the reaction of oADP with pyruvate kinase is therefore most consistent with the formation of a morpholine derivative.

The peptide containing the radioactive analog was isolated and sequenced (2). For

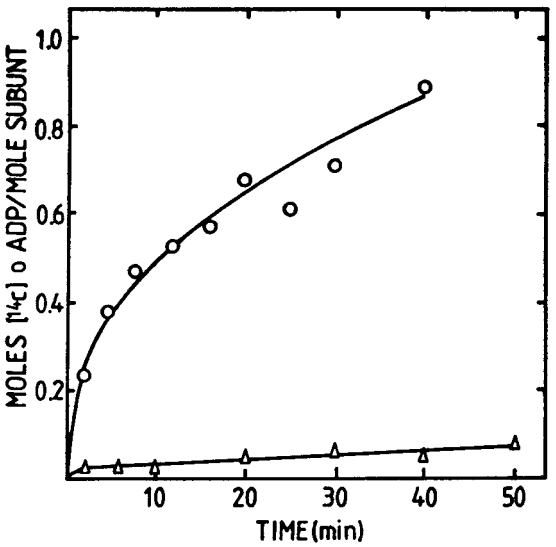

Fig 1. Time-course of the incorporation of [^{14}C]oADP into pyruvate kinase. Purified rabbit muscle pyruvate kinase (2.6 mg/ml) was incubated at 25° C with [^{14}C]oADP (0.3 mM) in cacodylate buffer (20 mM, pH 7.5): (Δ) in the presence of 25 mM ATP and 27 mM $MgSO_4$; (o) in the presence of 1 mM $MgSO_4$. At the indicated times 50 microliters aliquots were taken and placed immediately on a glass fiber disc which was put in cold 10% trichloracetic acid, washed as described by Corbin and Reimann (8) and then counted in a scintillation counter. From Bezares *et al* (2).

that purpose the enzyme was incubated with non-radioactive oADP in the presence of MgATP. Under these conditions the enzyme was not inactivated. After removal of the protective effectors, the enzyme was incubated with [^{14}C]oADP, and now labeling and inactivation occurred. Figure 2 shows that the incorporation of approximately one mole of the analog per subunit correlates with total loss of activity.

The modified enzyme was reduced, carboxymethylated, and digested with trypsin. The isolation of the oADP-peptide was performed by chromatography on Sephadex G-25 and HPLC. The purified peptide was sequenced by the automatic gaseous-phase Edman method. The complete sequence of a 34 amino acid residue peptide was obtained, small amounts of lysine were detected at position 25, but no radioactive label was found (2). The sequence obtained for the peptide was identical to a peptide previously isolated from bovine muscle pyruvate kinase labeled with trinitrobenzenesulfonate (21), and the ε-trinitrophenylated lysine at position 25 was the same lysine found in the oADP-labeled peptide. The same sequence was found between residues 341 and 374 in chicken muscle pyruvate kinase (28), and between residues 342 and 375 of the cat muscle enzyme (35). Regions of high homology to this peptide were also found in yeast pyruvate kinase between residues 313-337 (4), so as in residues 355-388 of the rat liver enzyme (29). The fact that this region is highly conserved from low eukaryotes to mammals strongly suggests that it is located in a site essential for catalysis, probably at or near the nucleotide binding site. Muirhead *et al* (35) based on X-ray crystallographic studies of the cat muscle enzyme, propose that residues 358 to 366 are close to the active site, but suggest no specific function for lysine 366 (the lysine equivalent to the residue labeled by us).

Our results show that in cases where the reaction between an oxidized nucleotide and an enzyme give a morpholine-like adduct, it is possible to isolate a labeled peptide if precautions are taken to avoid drastic reaction conditions. The label stays bound to the peptide during reduction, carboxymethylation, trypsin treatment, and chromatography in NH_4HCO_3 at pH 8.0, although it is lost during the sequencing process.

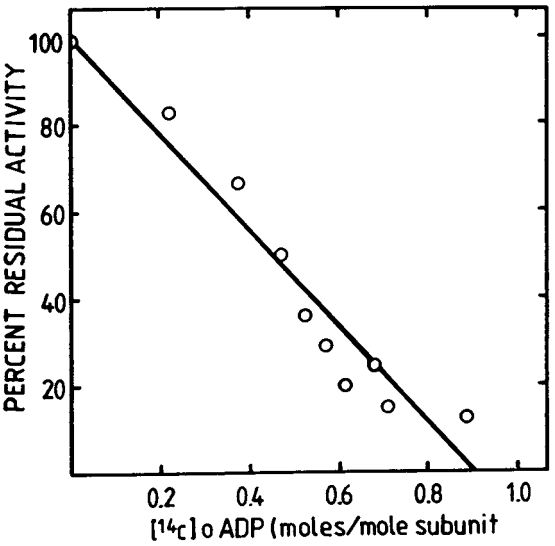

Fig 2. Correlation between inactivation of pyruvate kinase and incorporation of [^{14}C]oADP. Pyruvate kinase (2.6 mg/ml) was incubated at 25° C with [^{14}C]oADP (0.3 mM) in the presence of cacodylate buffer (20 mM, pH 7.5), and $MgSO_4$ (1 mM). At intervals, aliquots were withdrawn in order to follow the time course of the reaction: 10 microliters were used for enzyme activity measurements, and 50 microliters were taken for [^{14}C]oADP incorporation measurements according to the method of Corbin & Reimann (8). From Bezares *et al* (2).

When using oxidized nucleotides it should be kept in mind that these derivatives are not very stable. The half-life of oAMP at 20°C and pH 7.0 has been estimated at 35 hours (15); decomposition may produce free phosphate and also generate polymeric material. The stability is enhanced at lower pH or by cooling. Lowe & Beechey (32) prepared a decomposition product from oATP (which they called "compound II") by incubating oATP at pH 9.1 and 35°C for 22 hours. Compound II is formed in a ß elimination reaction, liberating the tripolyphosphate ion and retaining the aldehyde group. This compound is as reactive as oATP. In fact, the incubation of compound II with ATPase produced the inactivation of the enzyme at a faster rate than oATP. However, in contrast to oATP, no rate-saturation effect was seen, suggesting that the inactivation mechanism is different (32). Taking these facts into account, it is recommended that dialdehyde derivatives be used freshly prepared, and their purity be always checked by thin-layer chromatography on polyethyleneimine sheets (14) or by electrophoresis on cellulose plates (24). Otherwise, one may be dealing with different compounds.

In summary, the dialdehyde derivatives have been shown to be very useful site-specific reagents for a variety of ATP and NADH utilizing enzymes, and show the potential for more detailed structure-activity studies.

2. *Nucleoside polyphosphopyridoxals*

In recent years, a new class of aldehyde affinity labels directed to lysine residues in nucleotide binding sites has been designed. These are the nucleoside polyphosphopyridoxals, obtained by linking pyridoxal-5'-phosphate (PLP) to a nucleoside. PLP itself has been widely used as a group-specific reagent directed to lysine residues, and has shown in many cases to act as a real affinity reagent for phosphate binding sites, forming a non-covalent intermediary complex (13, 27). The usefulness of PLP as affinity label has been recently reviewed (46).

Uridine diphosphopyridoxal (UP_2PL) and adenine diphosphopyridoxal (AP_2PL) have been synthesized (41, 42). These compounds are structurally similar to the natural coenzymes, they are water soluble and negatively charged at neutral pH. They react with ε-amino groups of lysine to form Schiff's bases which can be stabilized by reduction with $NaBH_4$ UP_2PL produced the inactivation of glycogen synthase with reversible binding to the enzyme prior the covalent reaction, the enzyme being protected against inactivation by UDP-glucose and UDP. The modified and $NaBH_4$-reduced enzyme was carboxymethylated and digested with chymotrypsin and a peptide containing a labeled lysine was isolated (41).

The inactivation of yeast phosphoglycerate kinase, yeast alcohol dehydrogenase, yeast hexokinase and rabbit muscle pyruvate kinase by AP_2PL has also been reported. This reagent was found to bind more tightly and more specifically than PLP to these enzymes except rabbit muscle pyruvate kinase which was modified equally well by PLP or by AP_2PL (44).

Yeast hexokinase PII was inactivated by AP_2PL the binding of the reagent being enhanced by glucose. Moreover, ATP binds competitively with respect to AP_2PL. These facts suggest that modification occurs at the ATP binding site. The incorporation of one mole of reagent per mole of subunit was required to produce complete inactivation. From the modified and reduced enzyme subjected to trypsin digestion, a labeled peptide was isolated and Lys^{111} was identified as the labeled residue (45).

Rabbit muscle adenylate kinase has also been tested with AP_2PL. This reagent produced the inactivation of the enzyme. ADP, ATP and MgATP protected against the inactivation. Complete inactivation was estimated to be produced when one mole of the reagent was incorporated per mole of enzyme. From the modified enzyme, a labeled peptide was isolated and Lys^{21} was identified as the labeled residue (42).

Nucleoside polyphosphopyridoxals have been demonstrated to be useful reagents for the modification of nucleotide binding sites in proteins. The modified and reduced proteins are stable to peptide purification and sequencing procedures.

3. *Fluorosulfonylbenzoyl analogs*

Fluorosulfonylbenzoyl nucleotides include several compounds containing the purine and ribose structures and a reactive sulfonyl fluoride located in the phosphoryl region of the normal nucleotide. The first of this group of compounds synthesized was 5'-p-fluorosulfonylbenzoyladenosine (5'-FSBA) (36). This is an analog of ADP, ATP or NADH, the sulfonyl fluoride being located in a position corresponding to the γ-phosphoryl of ATP or to the nicotinamide ribose of NADH. This reactive group is capable of reacting with several amino acid residues including tyrosine, lysine, histidine, cysteine and serine (6). The derivatives obtained are unstable in aqueous solutions and they hydrolyze at low rate at neutral pH with release of fluoride ion. Because of the low stability and low solubility in aqueous solutions, they are usually prepared as stock solutions in organic solvents (dimethylformamide, dimethylsulfoxide).

As an example, the reaction of 5'-FSBA with rabbit muscle pyruvate kinase produced the inactivation of the enzyme (1). The inactivation proceeded with biphasic kinetics and the addition of dithiothreitol to the incubation mixture produced partial reactivation. In fact, the inactivation of the enzyme by 5'-FSBA includes three events: a fast reaction reactivable by dithiothreitol giving a partially active enzyme with 67% of residual activity, and two first-order reactions yielding fully inactive enzyme. One of the slow reactions was sensitive to dithiothreitol and the other was not. Only the dithiothreitol-insensitive component of the inactivation was proportional to the incorporation of about one mole of reagent per mole of enzyme subunit and was protected by ADP plus Mg^{+2}, suggesting the modification of an amino acid residue within the catalytic metal-nucleotide binding site. When the modified enzyme was digested with thermolysin and the mixture of peptides fractionated by HPLC, five peptides were isolated and identified by comparison with the known cat muscle pyruvate kinase sequence. Three of the modified residues were tyrosines (Tyr^{147}, Tyr^{160} and Tyr^{174}) and two were lysines (Lys^{299} and Lys^{366}). Since the loss of activity was directly proportional to the total incorporation of about 1 mole of reagent per mole of enzyme subunit, it was concluded that the modification of any of the five residues must result in an inactive enzyme. The most striking observation was that the modification of all the peptides was reduced in the presence of Mn-ADP or Mn-ATP (11).

A number of fluorosulfonylbenzoyl analogs of nucleotides have been synthesized by introduction or changes in position of different groups: the change of the fluorosulfonylbenzoyl group from the 5' to the 3' position results in 3'-FSBA (5); the introduction of guanine or inosine instead of adenine results in 5'-FSBG (37), or 5'-FSBI (48); derivatives containing the $1,N^6$ etheno group are fluorescent (26); those including the azido group are photoactivable (12). For detailed information about these compounds the reader may refer to Colman (7).

4. *Alkyl halides, haloacyl and haloketone derivatives of purine derivatives*

In general, alkyl halides are the most commonly used compounds for chemical modification of proteins. They react with several amino acid residues such as cysteine, lysine, histidine, methionine, aspartate and glutamate. Purine nucleotide derivatives have a negative charge at neutral pH which may be important for binding of the affinity label to the enzyme. They are also soluble in water and stable over a wide range of pH. A major limitation of these compounds is their low reactivity. As an example of using this type of reagents is the modification of NAD^+-dependent isocitrate dehydrogenase by adenosine 5'-(2-bromoethyl) phosphate. This reagent produces the inactivation of the enzyme in a time-dependent process with pseudo-first-order kinetics. ADP, an allosteric activator of the enzyme, protects against the loss of activity. Kinetic data show a non linear dependence of the rate constant on the reagent concentration, as expected for an affinity label (39).

Haloacyl derivatives are more reactive than alkyl halide ones. Reaction of haloacyl groups have been reported for cysteine, lysine, histidine, aspartate, glutamate and

tyrosine. The reaction products of these compounds give the carboxymethylamino derivatives which can be isolated and identified. For example, alcohol dehydrogenase from *Bacillus stearothermophilus* was modified with 4-(3 bromoacetyl pyridinio) butyldiphosphoadenosine, a structural analog of NAD^+, and a reactive cysteine was identified (20). Yeast and liver alcohol dehydrogenase were also tested with this reagent and both enzymes were inactivated with saturation kinetics and incorporation of one mole of reagent per mole of enzyme. Labeled peptides from both enzymes were purified, sequenced and the position of modified cysteines were determined (50). Several other haloacyl derivatives of nucleotides have been synthesized and tested on different enzymes (7).

In recent years a number of haloketones derivatives of nucleotides have been synthesized. These compounds are water-soluble, negatively charged at neutral pH and highly reactive. The bromoketo group may react with different nucleophiles found in proteins such as cysteine, histidine, tyrosine, aspartate and glutamate. In addition, the diketo moiety is capable of reacting with arginine residues. The synthesis and characterization of several 4-bromo-2,3 dioxobutyl derivatives of mercaptopurine nucleotides have been described and the bromoketo substituent has been located in different positions of the purine ring (7). Thus, the 2, 6 and 8 derivatives have been synthesized. These related compounds allow the systematic probing of different subregions within the purine binding site in an enzyme. For example, rabbit muscle pyruvate kinase was inactivated by 2, 6 and 8-bromodioxobutyl derivatives, and the metal-nucleotide offered effective protection against inactivation by the 2 and 6 substituent, whereas phosphoenolpyruvate and metal ion reduced the inactivation rate produced by the 8-bromodioxobutyl derivative (7).

Derivatives containing the bromoketo group in the carbon 5' of the ribose have also been prepared. Guanosine 5'-O-[S-(4-bromo-2,3-dioxobutyl)] thiophosphate (GMPS-BDB) and adenosine 5'-O-[S-(4-(4-bromo-2,3-dioxobutyl)] thiophosphate (AMPS-BDB) have the reactive 4-bromo-2,3-dioxobutyl group in the enol form in aqueous solution with a conformation similar to that of the substrates ADP plus PEP (bisubstrate analog). These compounds inactivated rabbit muscle pyruvate kinase in a pseudo-first-order reaction with saturation kinetics, the inactivation reaction being protected by PEP and K^+ or Mg^{+2} when using GMPS-BDB, or by divalent metal ions and nucleotides when using AMPS-BDB. After tryptic digestion of the modified enzyme, three labeled peptides were purified from the unprotected enzyme and one labeled peptide from the protected sample. Cys^{164} was identified as the nonessential residue labeled under protecting conditions and Cys^{151} and Cys^{48} were identified as the residues responsible for loss of activity; they react apparently in mutually exclusive reactions (47).

5. *Photoaffinity derivatives*

Photoaffinity derivatives form covalent bonds with enzymes only when the reaction is activated by light. Thus it is possible to study the reversible interaction between the enzyme and the reagent in the absence of light. On the other hand, the drawback of these compounds is that they usually react rapidly with the solvent with poor incorporation into the protein. In addition, the products of reactions are often not structurally defined or are unstable and difficult to isolate. These compounds react by several mechanisms such as insertion into C-H, O-H or N-H bonds. The principles of photoaffinity labeling are discussed by Schaefer in another chapter of this issue.

The most commonly used photoaffinity reagents to label nucleotide binding sites in enzymes are azido nucleotide analogs. The azido group has been incorporated directly in a nucleotide (9), or in a derivative such a fluorosulfobenzoyl adenosine (12), or periodate-oxidized nucleotide (23).

CONCLUDING REMARKS

The main experimental techniques currently used to elucidate the mechanism of action of enzymes are kinetic studies, chemical modification of amino acid side chains, site-

directed mutagenesis and X-ray crystallography. The information obtained from one approach should complement the results obtained from the others. Affinity labeling has proved to be a very useful tool for searching important amino acid residues located in active or allosteric sites of enzymes. This is the most popular technique of chemical modification and should continue to be an important primary tool for structure-function studies.

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