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Targeting enzyme sites in pyruvate kinase, glutamate dehydrogenase and glutathione S-transferase

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Abstract

Purine nucleotide analogs with reactive functional groups at various positions of the purine or ribose ring can be effective in mapping the active sites and regulatory sites of kinases and dehydrogenases. We have synthesized 5'-p-fluorosulfonylbenzoyl (FSB) derivatives of adenosine, guanosine, 8-azidoadenosine and the fluorescent 1,N^6-ethenoadenosine in which the electrophilic FSB moiety occupies the pyrophosphate region of the natural nucleotide. In addition, analogs with a 4-bromo-2,3-dioxobutylthio (BDB-T) moiety adjacent to the 2, 6 or 8 positions of the purine ring of nucleotides have been synthesized. These compounds bind to specific nucleotide sites in pyruvate kinase, glutamate dehydrogenase and other enzymes prior to covalently labeling those sites at amino acids such as cysteine, tyrosine, lysine, histidine, aspartic acid and glutamic acid. Affinity labeling experiments have been used directly to probe structure-function relationships in enzymes, as well as to rationally select targets for site-directed mutagenesis. We have also synthesized a reactive bromodioxobutyl derivative of the tripeptide glutathione. Studies of pyruvate kinase, glutamate dehydrogenase and glutathione S-transferase are described in this paper which illustrate the application of these compounds to affinity labeling of enzymic catalytic sites.

INTRODUCTION

One approach to locating the active site of an enzyme is to chemically modify an amino acid residue and then to correlate the extent of modification of that amino acid with the extent of loss of activity of the enzyme. It is, however, difficult to limit chemical modification of a protein to one or a few residues. We have chosen to exploit the specificity of the enzyme for its natural substrate to limit the extent of chemical modification to the region of the catalytic site. In this approach, a reagent is designed which is structurally similar to the normal substrate of the enzyme, but which also features a functional group capable of reacting covalently with many different amino acids. Such a compound is intended to mimic the substrate in forming a reversible enzyme-reagent complex analogous to the enzyme-substrate complex and, once bound at the specific substrate site, reacts irreversibly with an amino acid residue within that site. For several years, we have been designing purine nucleotide analogs with reactive groups at various positions of the purine or ribose ring in order to map the active sites and regulatory sites of purified dehydrogenases and kinases (refs. 1-3) and recently we have extended these approaches to other classes of purified enzymes.

Examples of such compounds are the fluorosulfonylbenzoyl derivatives of nucleosides shown in Fig.1. Compound (a) is 5'-p-fluorosulfonylbenzoyl adenosine (5'-FSBA) which might reasonably be considered as an analog of ADP, ATP or NADH. In addition to the adenine and ribose moieties, it has a carbonyl group adjacent to the 5'-position which is structurally similar to the first phosphoryl group of the naturally occurring purine nucleotides. If the molecule is arranged in an extended conformation, the sulfonyl fluoride moiety may be located in a position analogous to the terminal phosphate of ATP or to the ribose proximal to the nicotinamide ring of NADH. This sulfonyl fluoride moiety can act as an electrophilic agent in covalent reactions with several classes of amino acids, including tyrosine, lysine, histidine, serine and cysteine (refs. 1,3).

Structure (b) of Fig. 1 is 5'-p-fluorosulfonylbenzoyl guanosine, in which guanine replaces the adenine moiety in the first derivative. It might be anticipated that this purine nucleotide alkylating agent would be specifically directed toward GTP sites in proteins. Fig. 1 (c) is the fluorescent compound 5'-p-fluorosulfonylbenzoyl-1,N^6-ethenoadenosine. This nucleotide analog, with a fluorescence emission maximum at 412 nm, provides a means of introducing a covalently bound fluorescent probe into nucleotide sites in proteins. The final compound of Fig. 1, (d), is a bifunctional affinity label, 5'-p-fluorosulfonylbenzoyl-8-azidoadenosine, which contains both an electrophilic fluorosulfonyl moiety and a photoactivatable azido group. Following stoichiometric incorporation of reagent through the fluorosulfonyl at a specific site, photolysis of the tethered molecule can lead to reaction with amino acids adjacent to the residue which is initially labeled. This two-step reaction can help to elucidate the tertiary structure of the enzyme in the region of the nucleotide site.

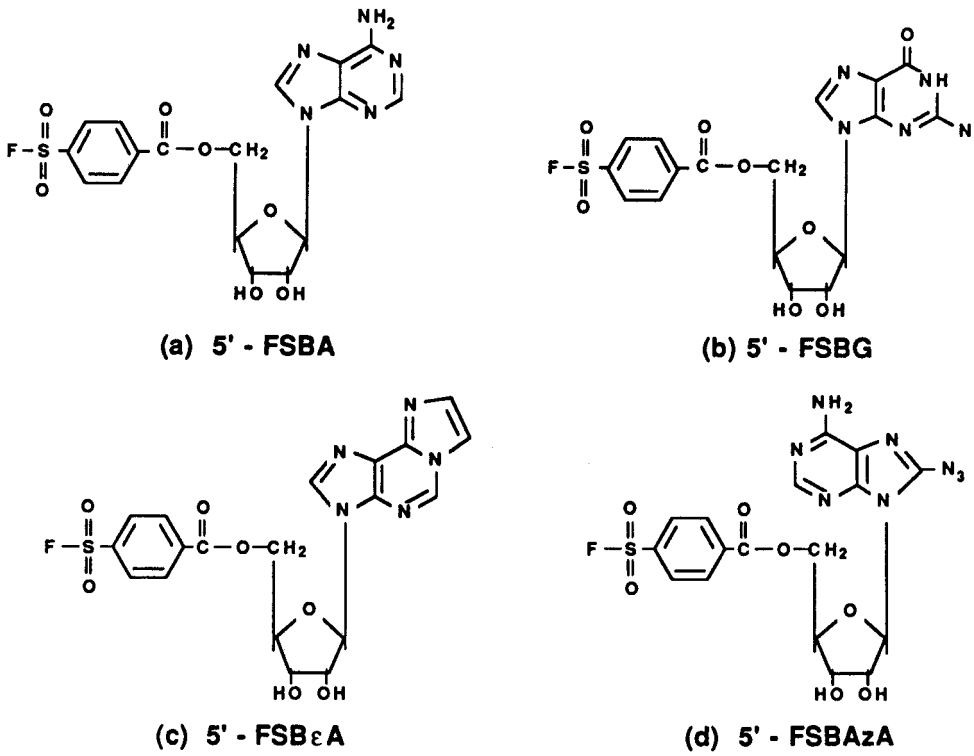
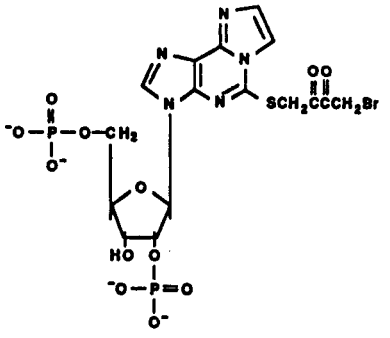
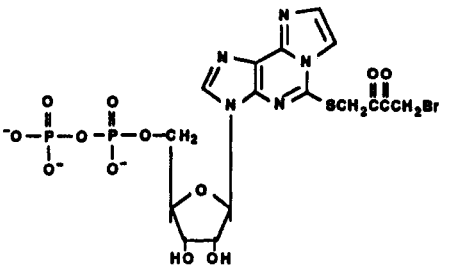


Fig. 1. Fluorosulfonylbenzoyl nucleosides: (a) 5'-p-fluorosulfonylbenzoyl adenosine; (b) 5'-p-fluorosulfonylbenzoyl guanosine; (c) 5'-p-fluorosulfonylbenzoyl-1,N^6-ethenoadenosine; and (d) 5'-p-fluorosulfonylbenzoyl-8-azidoadenosine.

Fig. 2 (b) illustrates a different type of nucleotide analog that we have synthesized: 2-[4-bromo-2,3-dioxobutylthio]-1,N^6-ethenoadenosine 5'-diphosphate (2-BDB-TA 5'-DP) (refs. 2,3). This compound is structurally similar to natural ADP, with its 5'-diphosphate. It is also water soluble and negatively charged at neutral pH. The bromoketo group is potentially reactive with several nucleophiles found in proteins, including cysteine, histidine, tyrosine, lysine, glutamic and aspartic acid; and the dioxo group lends the possibility of reaction with arginine residues. Furthermore, the compound features the fluorescent ethenoadenosine moiety. In addition, we have synthesized 2-, 6- and 8-bromodioxobutyl derivatives of adenosine 5'-diphosphate (refs. 2,3), which allow a systematic probing of the amino acid residues around the purine region of the ADP binding site of enzymes.

Each of these nucleotide derivatives contains a reactive functional group that is relatively indiscriminate in reactions with amino acids. In affinity labeling studies, the types of amino acid participants in the ligand binding site are frequently unknown. Using a non-specific functional group improves the likelihood of covalent reaction after the binding specificity is determined by the remainder of the structure of the affinity label.





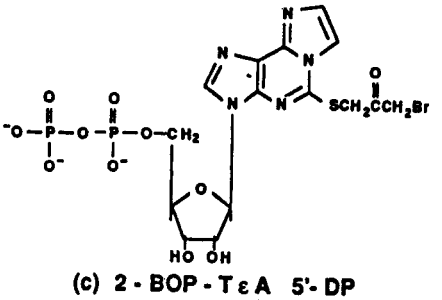


Fig. 2. Bromodioxobutyl nucleotides: (a) 2-[4-bromo-2,3-dioxobutylthio]-1,N^6-ethenoadenosine 2',5'-diphosphate; (b)2-[4-bromo-2,3-dioxobutylthio]-1,N^6-ethenoadenosine5'-diphosphate; and (c) 2-[3-bromo- oxopropylthio]-1, N^6 - ethenoadenosine 5'-diphosphate.

In this paper, several bromodioxo derivatives will be used to illustrate affinity labeling of three different types of enzymes: pyruvate kinase, glutamate dehydrogenase and glutathione S-transferase. For pyruvate kinase and glutathione S-transferase, these studies were used to identify the active site of the enzymes. In the case of the bacterial glutamate dehydrogenase, affinity labeling was used to indicate the region of the coenzyme binding site of the enzyme; we were then positioned to rationally choose target sites for mutagenesis, so that we could subsequently examine the properties of mutant glutamate dehydrogenase with particular amino acids replaced.

PYRUVATE KINASE

Pyruvate kinase is the key glycolytic enzyme which catalyzes the transfer of a phosphoryl group from phosphoenol pyruvate to ADP yielding pyruvate and ATP as the products. Considerable information is available now on the structure of this enzyme. Complete amino acid sequences are known for several isozymes, and the amino acid sequence of the cat muscle pyruvate kinase has been fitted to the 2.6 Å resolution electron density map (ref. 4). Affinity labeling with reactive nucleotide analogs offers a complementary technique for investigating the active site as it exists when the enzyme is in solution.

The first analog that we used is 2-[4-bromo-2,3-dioxobutylthio]-1,N^6-ethenoadenosine 5'-diphosphate (Fig. 2b). Incubation of rabbit muscle pyruvate kinase with 100-600 M 2-BDB-TεA 5'-DP resulted in a time-dependent inactivation of the enzyme. Biphasic inactivation kinetics were observed that could be described in terms of a fast initial phase of inactivation yielding a partially active enzyme with 25% residual activity, followed by a slower phase leading to totally inactive enzyme (ref. 5). Both phases exhibit a nonlinear dependence of the rate constant of inactivation on the reagent concentration, indicative of the initial formation of a reversible enzyme-reagent complex prior to irreversible modification. For the fast phase, a double reciprocal plot of $1/k_{obs}$ versus 1/[reagent] yields values of K_I = 133 μM and k_{max} = 0.193 min^{-1} at saturating concentrations of reagent. The slow phase has a k_{max} that is ten times lower (ref. 5).

Pyruvate kinase was incubated with 200 μM 2-BDB-TεA 5'-DP and the incorporation of reagent into the enzyme was measured at various times by quantitation of the organic phosphorus or fluorescence intensity. At 80 min., when the enzyme is 90% inactivated, about 1.7 mols of reagent/mol enzyme subunit is incorporated. Including either ADP + Mn^{++} or phosphoenol pyruvate + K^{+} + Mn^{++} in the reaction mixture caused a substantial reduction in the % inactivation at 80 minutes, as well as a decrease in the reagent incorporation to about 1 mol/mol enzyme subunit. These results indicate that 2-BDB-TεA 5'-DP reacts with two groups on the enzyme, one of which is at or near the active site. Since phosphoenol pyruvate is the most effective in protecting against inactivation, it seems likely that reaction occurs near the PEP binding site of pyruvate kinase.

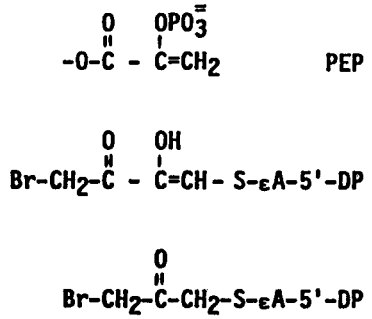


Fig. 3. Comparison of the structure of phosphoenolpyruvate (upper) with those of side chains of 2-BDB-TεA 5'-DP (middle) and 2-BOP-TA 5'-DP (lower). Reproduced from Reference 5 with permission.

NMR evidence indicates that the diketo groups of bromodioxobutyl-nucleotides exist predominantly in enolized form when they are dissolved in aqueous solution, as shown in Fig. 3. The reagent 2-BDB-TεA 5'-DP thus resembles phosphoenolpyruvate. In contrast, a monoketo compound, such as 2-[3-bromo-oxopropylthio]-1,N^{6}-ethenoadenosine 5'-diphosphate (shown in Fig. 2c), does not enolize in aqueous solution. If the enolate directs the reagent to the PEP binding site, the bromooxopropyl derivative should be unreactive. In fact, this proved to be the case. Incubation of the enzyme with 200 μM 2-BOP-TεA 5'-DP for 80 min resulted in only 14% loss of enzyme activity, whereas the bromo*dioxo* derivative caused 90% inactivation under the same conditions.

In order to identify the amino acids which were reacting, modified enzyme was prepared by incubating pyruvate kinase with 2-BDB-TεA 5'-DP either in the absence or in the presence of phosphoenol pyruvate, K^{+} and Mn^{++}. The isolated enzymes, freed of reagent, were reduced with [^{3}H]-$NaBH_4$ to introduce the radioactive tag. The enzymes were then digested with trypsin, and the digests were purified by chromatography on a phenylboronate agarose column followed by reverse phase HPLC using a trifluoroacetic acid solvent system.

Tryptic digests of the inactive modified enzyme yielded two labeled peptides with the following sequences:

I. Asn-Ile-Cys^{164}-Lys

II. Ile-Thr-Leu-Asp-Asn-Ala-Tyr^{147}-Met-Glu-Lys

Only peptide II was isolated from digests of pyruvate kinase that had been modified in the presence of phosphoenol pyruvate and therefore retained most of its activity. Since Cys-164 is labeled equally whether the enzyme is active or inactive, it cannot be essential for activity. In contrast, modification of Tyr-147 by 2-BDB-TεA 5'-DP is associated with inactivation.

The structure of pyruvate kinase in the crystalline state has been determined by Muirhead et al. (ref. 4). The active site is thought to lie in a pocket between Domain A and Domain B, with Tyrosine-147 being located at the entrance to the active site. Specific reaction of Tyrosine-147 with 2-BDB-TεA 5'-DP causes loss in activity. Cysteine-164 is an internal residue in Domain B. Its position outside the active site is consistent with its modification having little effect on catalysis.

As a second type of nucleotide analog for probing the active site of pyruvate kinase, we synthesized 8-[4-bromo-2,3-dioxobutyltho]adenosine di- and triphosphate (8-BDB-TATP)(ref. 6). We thought that the placement of the bromoketo group adjacent to the 8 position of the adenine ring might allow the compound to react differently from 2-BDB-TεA 5'-DP. Incubation of pyruvate kinase with 175 μM 8-BDB-TADP or 8-BDB-TATP at pH 7.0 and 25° caused biphasic inactivation. The reactions of the two analogs are similar, but the rate constant for the triphosphate derivative is about three times greater than that of the diphosphate compound.

Incorporation of reagent into the enzyme was measured at various times during the incubation by quantitation of the organic phosphorus. The triphosphate derivative is more specific, exhibiting lower incorporation for a higher degree of inactivation. At 80 minutes, 2.05 mol of 8-BDB-TATP per mol of enzyme subunit is incorporated when the enzyme has lost 97% of its original activity. Protection against inactivation by 8-BDB-TATP is provided by various combinations of substrates. In the presence of substrates, the enzyme loses only 15-20% of its activity, and the incorporation of reagent is reduced to about 1 mol/mol enzyme subunit. These results suggest that 8-BDB-TATP also reacts with two groups on the enzyme, one of which is at or near the PEP site.

The residues modified by 8-BDB-TATP have been identified by reduction of the modified enzyme with [^{3}H]-sodium borohydride, carboxymethylation of the free cysteines, digestion with trypsin and purification of the resultant peptides on a phenylboronate agarose column followed by HPLC (ref.7). Two cysteine-modified peptides were isolated from the digest of inactive enzyme:

I. Asn-Ile-Cys^{164}-Lys

III. Cys^{151}-Asp-Glu-Asn-Ile-Leu-Trp-Leu-Asp-Tyr-Lys

Reaction in the presence of the protectants phosphoenol pyruvate, K + Mn^{++} yielded Asn-Ile-Cys-Lys as the only labeled peptide, with Cys-164 as the modified residue. This is the same residue which reacts with the 2-substituted nucleotide analog under protecting conditions. Although Cys-164 is clearly not required for activity and is internal, it appears to be favorably positioned to react with nucleotide analogs. The major residue responsible for inactivation of pyruvate kinase by 8-BDB-TATP seems to be Cys-151. This result contrasts with the finding that in the case of 2-BDB-TεA 5'-DP, it is modification of Tyrosine-147 that is responsible for loss in enzyme activity. The distance between the α-carbons of Cys-151 and Tyr-147 in the crystal structure of pyruvate kinase is 8.9Å. Since the target residues of the two affinity labels are approximately 9Å apart, the question arises as to whether, if the nucleotide moieties were superimposed, the reactive -CH_2Br groups would be situated 9Å apart.

Fig. 4 compares the structures of 2-BDB-TεA 5'-DP, shown in (a) in the anti conformation about the purine-ribose bond, and 8-BDB-TATP, pictured in (b) in the syn conformation about the purine-ribose bond. In the middle, the two structures are superimposed so that the riboses and phosphates coincide. Although a range of distances between the two -CH_2Br groups is possible, it *is* energetically permissible to arrange the compounds on the enzyme such that the distance between the reactive groups coincides with the 9Å distance between Cys-151 and Tyr-147 (ref. 7). If the purines, riboses and phosphates of the two compounds bind similarly to the enzyme, the experimental results from affinity labeling are thus consistent with the atomic positions assumed by the enzyme in the crystalline state.

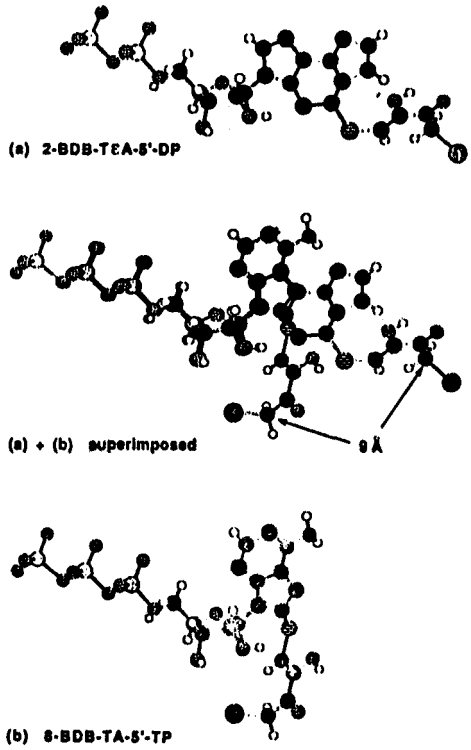


Fig. 4. Comparison of the structures of (a) 2-BDB-TεA 5'-DP and (b) 8-BDB-TATP. Reproduced from Reference 7 with permission.

GLUTAMATE DEHYDROGENASE

I am going to turn now to our studies of the enzyme glutamate dehydrogenase, which catalyzes the oxidative deamination of the amino acid glutamate to form α-keto glutarate and ammonia. Glutamate dehydrogenase is widespread in nature exhibiting a coenzyme specificity which depends on the source of the enzyme. The mammalian enzyme, as exemplified by the bovine liver enzyme that we have been studying for many years, can utilize both NAD and NADP, while the enzyme from microorganisms is specific either for NAD or for NADP. In addition, while the mammalian enzyme is highly regulated by a variety of nucleotides, the enzyme from microorganisms is not an allosteric enzyme. Glutamate dehydrogenase is composed of six identical subunits, with a molecular weight of 45,000 and 447 amino acids in each subunit for the *Salmonella* enzyme.

The gene from *Salmonella typhimurium* encoding glutamate dehydrogenase has been cloned and sequenced (ref. 8). The enzyme has been overexpressed in *E. coli,* making it a convenient source of bacterial glutamate dehydrogenase, and raising the possibility of engineering new forms of the enzyme by site-directed mutagenesis. Furthermore, a crystallographic group in Sheffield, England has been studying the structure of crystals of a related bacterial glutamate dehydrogenase from *Clostridium symbiosum* (ref. 9). Affinity labeling can indicate, for an enzyme in solution, that there is proximity between the modified amino acid and the natural ligand which is mimicked by the affinity reagent; it does not prove that the target amino acid makes a direct contribution to catalysis, regulation or binding. However, the possibility of a direct role for a particular amino acid which is suggested by either crystallographic data or affinity labeling can be explored further by site-directed mutagenesis. The bacterial glutamate dehydrogenase system offers the possibility of using the complementary techniques of crystallography, affinity labeling and site-directed mutagenesis to examine structure-function relationships. We initiated our exploration of the *Salmonella* glutamate dehydrogenase by affinity labeling studies, because we considered that these might provide a rational basis for the choice of target positions for site-directed mutagenesis experiments. Since the enzyme requires $NADP^+$, we first selected a reactive nucleotide analog with a 2'-phosphate: 2-BDB-thio-1,N^6-ethenoadenosine-2',5'-DP^8, shown in Fig. 2a, although we later carried out similar studies with the adenosine analog (ref. 10).

Incubation of the enzyme with 200 μM 2-BDB-TεA 5'-DP causes a time-dependent inactivation to yield a partially active enzyme of 40% residual activity. Neither additional reagent nor increases in time led to further inactivation, demonstrating that this was the limit of the reaction for this enzyme. The rate of inactivation exhibits a nonlinear dependence on the reagent concentration, indicating that 2-BDB-TεA 2',5'-DP binds reversibly to the enzyme prior to the irreversible reaction.

Complete protection against inactivation was provided by the specific coenzymes NADPH, NADP or the coenzyme fragment 2'-phospho-adenosine diphosphoribose, but not by NADH or the substrate α-ketoglutarate. Thus, reaction was directed to the region of the coenzyme binding site.

The amount of reagent incorporated as a function of time of incubation was measured from the fluorescence of bound 2-BDB-TεA 2',5'-DP yielding up to about 1 mole incorporated per peptide chain. The incorporation of reagent is decreased to 0.5 mol/peptide chain in the presence of NADPH, when the enzyme retains full activity. These results indicate that 2-BDB-TADP functions as an affinity label of the coenzyme binding site and that specific reaction occurs at only about 0.5 sites per enzyme subunit or 3 sites/enzyme hexamer (ref. 8). Glutamate dehydrogenase from several species has been shown to be organized as a dimer of trimers. In this case, modification and inactivation of one trimer must indirectly diminish the activity of the second trimer and decrease below the level of detection the reaction rate of that second trimer with 2-BDB-TεA 2',5'-DP.

Two major tryptic peptides modified by the reagent were isolated and identified: a pentapeptide including Cys-283 and Glu-284; and an 11-membered peptide with a modified Glu-153. In the presence of NADPH, which completely prevents inactivation, only the 11-membered peptide was labeled. The results indicate that modification of the pentapeptide causes loss of activity (ref. 8). However, we did not isolate directly the modified residue and initially there was some uncertainty as to whether the cysteine or the glutamate was the actual target of 2-BDB-TεA 2',5'-DP.

In order to examine these possibilities site-directed mutagenesis was used to engineer and then to express in *E. coli* three mutant *Salmonella* glutamate dehydrogenases: one in which glutamate was changed to the non-nucleophilic glutamine at position 284, a second in which the cysteine was changed to the non-reactive isoleucine at position 283, and a double mutant in which both amino acids 283 and 284 were changed (refs. 10,11). The substitution of isoleucine for cysteine was chosen because isoleucine is not a nucleophile and is found in the corresponding position in the *E. coli* enzyme. Replacement of glutamic acid by glutamine was selected to eliminate the nucleophilic character of the residue while retaining its size, in an attempt to avoid altering the conformation of the enzyme. We found that the glutamine mutant reacts with 2-BDB-TεA 2',5'-DP at about the same rate as the original wild type enzyme, demonstrating that Glu-284 could not be the target of 2-BDB-TεADP. In contrast, in the two mutant *Salmonella* glutamate dehydrogenases in which cysteine had been converted to isoleucine, there is no inactivation by added 2-BDB-TεADP. These results identify Cys-283 as the reaction site of this nucleotide analog (refs. 10,11).

We have purified to homogeneity these three mutant enzymes. The striking result is that they all have about the same specific activity as the wild type enzyme and exhibit the same K_m values for the 3 substrates NADPH, NH_4Cl and α-keto-glutarate. Since the mutant enzymes are fully active, Cys-283 cannot be required for catalysis. However, we consider that the results of the affinity labeling experiments locate Cys-283 within the coenzyme binding site. The 2-[4-bromo-2,3-dioxobutylthio]-1,N^6-ethenoadenosine 2',5'-diphosphate probably binds to the enzyme at the coenzyme binding site. If a nucleophilic amino acid is available, it then reacts covalently, thereby permanently occupying the coenzyme binding site, preventing further catalysis and inactivating the enzyme. Support for this conclusion comes from examination of the crystal structure of the glutamate dehydrogenase of *Clostridium symbiosum* (ref. 9). The two bacterial enzymes exhibit 64% sequence homology, which has made it possible to align the sequence of the *S. typhimurium* enzyme with the structure of the *C. symbiosum* enzyme (D. W. Rice, personal communication). The loop 282-286 is indeed located close to the bound coenzyme in the general region of the adenine ribose and pyrophosphate moieties.

Further examination of the crystal structure suggested that the Lys at position 286 might be closer to the phosphate binding region of the coenzyme binding site. Accordingly, we next prepared and studied a set of mutants with substitutions at this position. Lysine-286 was replaced by the positively charged arginine, by neutral glutamine and by negatively charged glutamic acid in order to assess the effect of the amino acid charge on coenzyme binding, catalysis and specificity. The mutant enzymes were prepared by site-directed mutagenesis, expressed in *E. coli* and then purified to homogeneity and characterized (ref. 12).

TABLE I: Determination of Km (NADPH) and kcat for Wild Type and Mutant Glutamate Dehydrogenases (ref. 12)

Enzyme	K_m (NADPH) (μM)	k_{cat} (NADPH) min^{-1}	Efficiency k_{cat}/K_m ($\mu M^{-1} min^{-1}$)
Wild Type	9.8	11.3	1200
Lys 286 Arg	30	18.1	610
Lys 286 Gln	66	13.8	210
Lys 286 Glu	280	11.8	42

All three mutants exhibited elevated K_m values for the coenzyme NADPH, as shown in Table I. The smallest change is seen for the arginine mutant in which the positively charged arginine was substituted for the positively charged lysine. Substitution of the neutral amino acid glutamine caused a greater increase in K_m for NADPH. However, replacement of the lysyl residue by the negatively charged glutamate at position 286 resulted in a 30-fold increase in the K_m for NADPH. This observation is consistent with our postulate that Lys-286 is normally involved in coenzyme binding. In contrast, the K_m values for the other substrates, ammonium chloride and α-ketoglutarate do not differ appreciably between the wild type and mutant enzymes.

At high and saturating concentrations of the coenzyme and substrates, the maximum velocity of the enzyme is obtained, and this is expressed in Table I as k_{cat}. The wild type and mutant enzymes have similar values of k_{cat} (ref. 12). The last column in Table I assesses the efficiency of the enzyme, which is defined as k_{cat}/K_m. The k_{cat}/K_m values show that the decreasing efficiency in the mutant enzymes, particularly that of the glutamate mutant, is attributable to the increase in K_m for NADPH.

The coenzyme NADH is structurally the same as NADPH, except that it lacks the negatively charged phosphate at the 2'-position. Although the *Salmonella* glutamate dehydrogenase is generally considered to be NADPH-specific, we were able to detect activity with NADH and to determine a K_m for NADH. With NADH as coenzyme, the k_{cat} is 10-fold lower and K_m is 200-fold higher than for NADPH as the coenzyme (ref. 12). Table II shows that, in contrast to the major differences in K_m for NADPH, wild type and mutant enzymes have similar K_m values for NADH. Since the efficiency of the mutant enzymes decreases for NADPH as coenzyme, but does not change appreciably when NADH is used as coenzyme, there is a decrease in the relative efficiency of the

TABLE II : Comparison of NADPH and NADH as Coenzymes for Wild Type and Mutant Glutamate Dehydrogenases (ref. 12)

Enzyme	K_m (NADH) (μM)	k_{cat}/K_m ($min^{-1} \mu M^{-1}$)		
		NADPH	NADH	NADPH/NADH
Wild Type	2000	1200	0.62	1900
Lys 286 Arg	1800	610	1.60	380
Lys 286 Gln	2100	210	1.10	200
Lys 286 Glu	2100	42	0.46	91

mutant enzymes for NADPH as compared to NADH, and the mutants become less specific for coenzyme. These results suggest that the positively charged residue Lysine-286 normally has a role in the binding of the 2'-phosphate group of NADPH which makes the enzyme more specific for this coenzyme.

Two additional reactive nucleotide analogs were synthesized in my laboratory which have been used recently to identify regulatory sites in the more complex bovine liver glutamate dehydrogenase. The bifunctional affinity label, 5'-p-fluorosulfonylbenzoyl-8-azidoadenosine, shown in Fig. 1 (d), reacts with glutamate dehydrogenase in a 2-step process: the initial dark reaction occurs through the fluorosulfonyl group and yields an enzyme which is modified at a specific NADH regulatory site (ref. 13). We have identified Tyrosine-190 as the predominant reaction site (ref. 14). Upon photolysis of the modified enzyme, about 25% of the covalently bound reagent becomes crosslinked to another part of the enzyme. We have recently ascertained that the predominant target amino acids are Leucine-477 and Arginine-478 near the C-terminus of the enzyme (ref. 14). This result indicates that, in order to be crosslinked by 5'-p-fluorosulfonylbenzoyl-8-azidoadenosine, Tyrosine-190 and Leu-477/Arg-478 must be a maximum of 14 Å apart in the folded structure of the enzyme (ref. 14).

The second new reactive nucleotide that we prepared is guanosine 5'-O-[S-(3-bromo-2-oxopropyl)]thiophosphate or GMPS-BOP, shown in Fig. 5 (a). This compound preserves the guanine and ribose and adds an alkylating group at a position equivalent to the pyrophosphoryl region of the natural nucleotide. The new analog is negatively charged at neutral pH and molecular modeling indicates that its size is equivalent to that of GTP [shown in Fig. 5 (b)]. We have recently completed a study of the covalent labeling of an allosteric site of bovine liver glutamate dehydrogenase by GMPS-BOP (ref. 15).

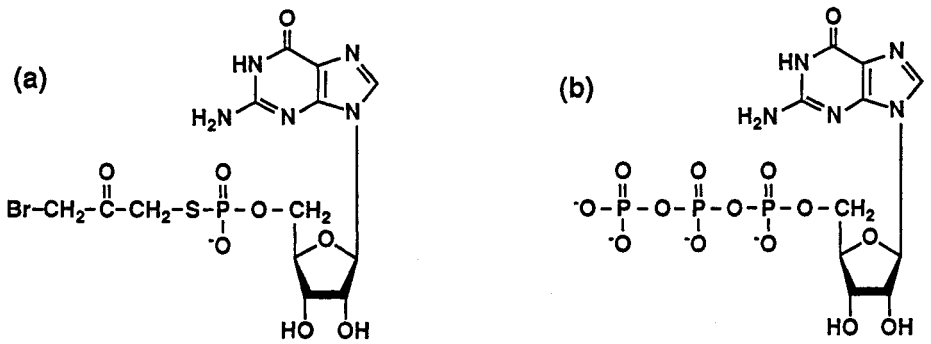
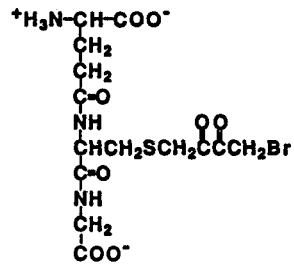


Fig. 5. Schematic structures of (a) GMPSBOP and (b) GTP.

GLUTATHIONE S-TRANSFERASE

The third type of enzyme that will be used to illustrate the approach of affinity labeling are the glutathione S-transferases, which constitute a family of enzymes important in the detoxification of xenobiotics or foreign chemicals. These enzymes catalyze reactions in which the thiol of glutathione undergoes nucleophilic addition to electrophilic substrates; the product is a glutathione conjugate of the xenobiotic compound which can then be excreted. These cytosolic enzymes are dimers with subunit molecular weights of 25,000-28,000 which, on the basis of isoelectric point, substrate specificity and primary sequence similarity, can be divided into at least three classes: α, μ and π (refs. 16,17). Crystal structures have now been published for representatives of the π and μ class of enzymes (refs. 18,19). Our aim in initiating affinity labeling studies was to obtain information on the active site of the enzyme in solution that would be complementary to the crystallographic studies.

We have synthesized the bromodioxobutyl derivative of glutathione, shown in Fig. 6, in which the reactive side chain is linked to the glutathione backbone through the original -SH of cysteine (ref. 20). The expectation was that the compound S-(4-bromo-2,3-dioxobutyl)glutathione [S-BDB-G] would bind to glutathione S-transferases since it is known that S-alkyl derivatives of glutathione, such as S-hexylglutathione, are effective competitive inhibitors of the enzyme.



S-BDB-G

Fig. 6. Structure of S-(4-Bromo-2,3-dioxobutyl)glutathione (S-BDB-G).

Rat liver isoenzyme 3-3 is a member of the μ class of glutathione S-transferases. Incubation of the 3-3 isoenzyme at pH 6.5 with 200 μM S-BDB-G results in a time-dependent total inactivation (ref. 21). The rate constant for inactivation exhibits a nonlinear dependence on reagent concentration as measured from 50-900 μM. This result suggests that prior to irreversible modification, a reversible enzyme-reagent complex is formed. This type of initial formation of an enzyme-reagent complex is expected for an affinity label and accounts for the relative specificity of such reagents. A K_I value of 120 μM can be calculated with a maximum rate constant of 0.073 min^{-1} estimated at saturating concentrations of reagent.

Complete protection against inactivation by S-BDB-G was provided by glutathione analogs with hydrophobic side chains of at least three carbons (i.e., S-propyl-, S-butyl-, S-pentyl- and S-hexylglutathione), but not by S-methylglutathione. These results indicate that the reaction target of S-BDB-G is the active site of the enzyme, probably the sub-site normally occupied by the electrophilic (hydrophobic) substrate.

Incubation of glutathione S-transferase with radioactive S-BDB-G leads to a time-dependent incorporation with 2 moles of reagent incorporated when the enzyme is completely inactivated. In the presence of the protecting substrate analog, S-hexylglutathione, the highest incorporation measured was 1 mole of reagent per mole of subunit, and the enzyme retained all of its activity (ref. 21). These results suggest that reaction occurs at one amino acid which is not essential for enzyme function and at a second amino acid which is important for enzymatic activity.

Enzyme modified with radioactive reagent in the absence or the presence of S-hexylglutathione was digested with trypsin and the resulting peptides were separated by HPLC. The chromatograms are shown in Fig. 7. Two major radioactive peaks (I and III) are observed in the digest from the inactive enzyme (Fig. 7, top panel). The lower panel shows the contrasting chromatogram derived from the active enzyme modified in the presence of S-hexylglutathione: Peak III is missing. Therefore, the peptide contained in this radioactive peak must be the one whose modification is correlated with inactivation.

The purified peptides were subjected to gas phase sequencing with the following results:

I. Lys-His-His-Leu-Cys^{86}-Gly-Glu-Thr-Glu-Glu-Glu-Arg

II. Lys-His-His-Leu-Cys^{86}-Gly-Glu-Thr-Glu-Glu-Glu-Arg-Ile-Arg

III. Met-Gln-Leu-Ile-Met-Leu-Cys-Tyr^{115}-Asn-Pro-Asp-Phe-Glu-Lys

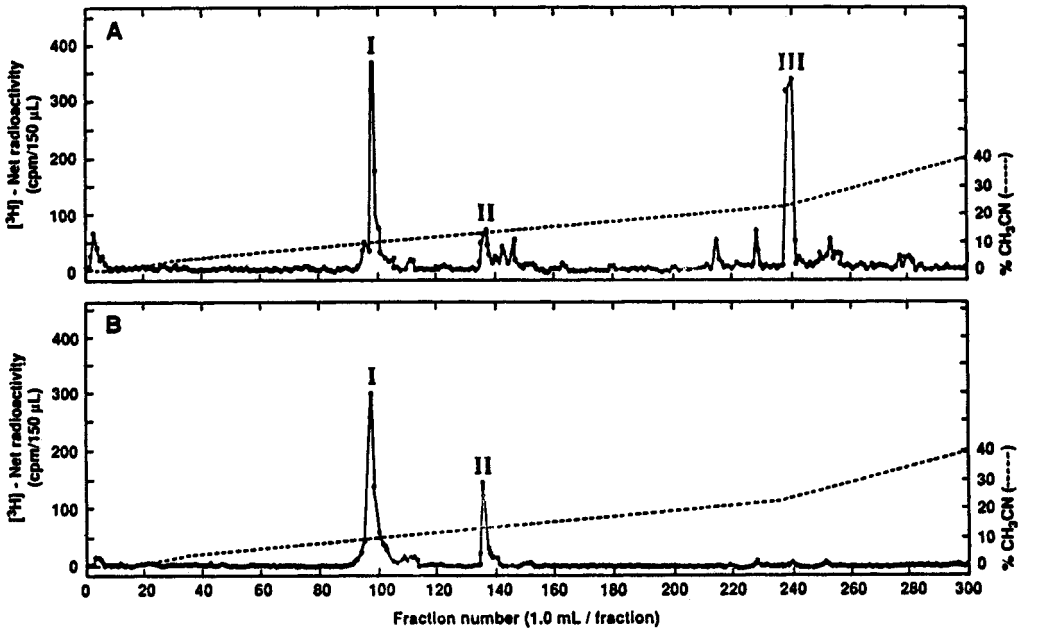


Fig. 7. Fractionation by reverse phase HPLC of tryptic digest of glutathione S-transferase isoenzyme 3-3. Peptides resulting from the modification of glutathione S-transferase, isoenzyme 3-3, by 200 μM [^{3}H]S-BDB-G were separated on a C_{18} column equilibrated with 0.1% trifluoroacetic acid using an acetonitrile gradient. (A) Distribution of radioactivity in the digest of modified enzyme prepared in the absence of substrate analog. (B) Distribution of radioactivity in the digest of enzyme modified in the presence of 5 mM S-hexylglutathione. Reproduced from Reference 21 with permission.

The peptides of Peaks I and II contained the same modified amino acid, Cys-86; these peptides differ only by 2 additional amino acids at the C-terminal end of Peptide II. Since Cys-86 is labeled when the enzyme is active or inactive, this amino acid is not essential to the enzymatic activity. Peak III contains a peptide in which Tyrosine-115 has been modified. Since this peptide is labeled only when the enzyme is inactive, reaction of S-BDB-G with Tyr-115 is responsible for inactivation of glutathione S-transferase (ref. 21).

The structure of crystalline rat liver glutathione S-transferase, isoenzyme 3-3, has recently been determined by x-ray diffraction (ref. 19). The distance between the sulfur of glutathione (bound in the active site cleft) and the oxygen of Tyrosine-115, which we have modified, has been measured by Armstrong as 7.5Å (Armstrong, R. M. personal communication). This distance is close to that expected to be occupied by the bromodioxobutyl side chain of S-BDB-G (6.8 Å is the distance between the sulfur and the bromo of -CH_2Br). This analysis thus supports our conclusion that the critical Tyrosine-115 of the 3-3 isozyme is located in the hydrophobic substrate binding portion of the enzyme's active site where its major function is to facilitate binding of the non-glutathione substrate through hydrophobic interactions.

Among the glutathione S-transferases within a given family of isozymes there is a high degree of sequence homology, but between families there is relatively little sequence similarity. The extent of the differences between isozyme sequences also appears to correlate with differences in substrate specificity. In addition to our study of the modification of the 3-3 isozyme of rat liver glutathione S-transferase (ref. 21) by S-BDB-G, we have examined the reaction of the same affinity label with two other isoenzymes of glutathione S-transferase: the 4-4 and 1-1 isozymes. The 4-4 enzyme is a member of the same family and is highly homologous to the 3-3 enzyme. The results are summarized in Table III. Not surprisingly, S-BDB-G inactivates the 4-4 isoenzyme (ref. 20) concomitant with labeling of the same residue, Tyr-115, as in the case of the 3-3 isozyme. This result indicates that the active sites of the 3-3 and 4-4 isoenzymes of glutathione S-transferase are very similar indeed, at least in the region occupied by the reactive BDB group of the reagent, which is likely that of the hydrophobic substrate.

TABLE III: Amino Acid Residue of Rat Liver Glutathione S-Transferase Which is Labeled by S-BDB-G and Which is Critical for Activity (ref. 20-22)

Isozyme	Family	Amino Acid Labeled
3-3	Mu	Tyr^{115}
4-4	Mu	Tyr^{115}
1-1	Alpha	Cys^{111}

In the case of the 1-1 isozyme, which is a member of a different family of glutathione S-transferases, S-BDB-G also inactivates the enzyme in a specific manner at the active site (ref. 22). However, the mode of binding of S-BDB-G and the critical amino acid labeled is distinct: Cys-111 is modified. Clearly the active site of the alpha family is distinguishable from that of the mu family.

CONCLUDING REMARKS

This paper has presented a representative sampling of the types of studies being conducted in my laboratory involving nucleotide and glutathione affinity labels. We are hopeful that these various analogs will not only be useful for our own experiments, but that they will also be valuable to other laboratories in exploring specific sites in a variety of proteins. The 5'-p-fluorosulfonylbenzoyl adenosine, which was the first of the reactive nucleotide analogs that we described, has already been found to yield specific labeling of NAD sites in several dehydrogenases and reductases (refs. 1,3). It has also labeled ATP or ADP binding sites in a large number of kinases and synthetases, in addition to providing an effective handle for examining an ADP receptor protein of platelet membranes. Furthermore, it has modified specific nucleotide sites in such diverse proteins as the ATPases, actin, myosin, luciferase and oxo-prolinase. In all, about 50 proteins have been studied using this compound (ref. 3). We anticipate that the FSB derivatives of guanosine, ethenoadenosine and azidoadenosine, as well as the new bromodioxobutyl derivatives will similarly have widespread applications to the elucidation of active sites and nucleotide binding sites in enzymes and receptors.

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