form of acetaldehyde was observed at a concentration as high as 0.4 mM.

These experiments proved that, for liver alcohol dehydrogenase, the carbonyl form is indeed the active substrate and that the catalysis of the dehydration of the hydrated form of acetaldehyde by the enzyme is not observable. This agrees with the general picture which emerges from the studies of carbonyl reducing enzymes. On the other hand, the hydrated form of acetaldehyde did not influence the kinetics of reduction of the carbonyl form, excluding the formation of strong complexes of the type enzyme–coenzyme–hydrated form. This is in contrast with the behavior of the hydrated form of trifluoroacetaldehyde which is a competitive inhibitor with respect to ethanol and thus binds to the enzyme–NAD^+ complex. Since no inhibition for the acetaldehyde hydrate has been detected, the trifluoromethyl group must tighten the binding. Such behavior is reminiscent of the increased binding observed on replacement of an acetyl by a trifluoroacetyl group (Renaud et al., 1978).

References

Baici, A., & Luisi, P. L. (1977) *J. Mol. Biol. 114*, 267.
Bell, R. P. (1966) *Adv. Phys. Org. Chem. 4*, 1.
Bell, R. P., Rand, M. H., & Wynne-Jones, K. M. A. (1956) *Trans. Faraday Soc. 52*, 1093.
Bränden, C. I., Jörnvall, H., Eklund, H., & Furugren, B. (1975) *Enzymes, 3rd Ed. 11*, 104.
Dalziel, K., & Dickinson, F. M. (1965) *Nature (London) 206*, 255.
Dixon, M., & Webb, E. C. (1964) *The Enzymes*, p 281, Academic Press, New York.
Fridovich, I. (1966) *J. Biol. Chem. 241*, 3126.
Guthrie, J. P. (1975) *Can. J. Chem. 53*, 898.
Hall, R. L., & Kezdy, F. J. (1970) *Arch. Biochem. Biophys. 138*, 551.
Husted, D. R., & Ahlbrecht, A. H. (1952) *J. Am. Chem. Soc. 74*, 5422.
Kurz, J. L. (1967) *J. Am. Chem. Soc. 89*, 3524.
Kvassmann, J., & Pettersson, G. (1976) *Eur. J. Biochem. 69*, 279.
Lienhard, G. E., & Anderson, F. H. (1967) *J. Org. Chem. 32*, 2229.
Müller-Hill, B., & Wallenfells, K. (1964) *Biochem. Z. 339*, 349.
Naylor, J. F., III, & Fridovich, I. (1968) *J. Biol. Chem. 243*, 341.
Pocker, Y., & Meany, J. E. (1967) *J. Phys. Chem. 71*, 3113.
Renaud, A., Dimicoli, J. L., Lestienne, P., & Bieth, J. (1978) *J. Am. Chem. Soc. 100*, 1005.
Tienhaara, R., & Meany, J. E. (1973) *Biochemistry 12*, 2067.

Affinity Labeling of an Allosteric GTP Site of Bovine Liver Glutamate Dehydrogenase by 5′-*p*-Fluorosulfonylbenzoylguanosine†

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ABSTRACT: In the presence of DPNH, native glutamate dehydrogenase binds, with markedly different affinities, 2 mol of the allosteric inhibitor GTP per peptide chain. In contrast, only 1 mol of GTP is bound in the absence of coenzyme. Incubation of enzyme with the guanosine nucleotide analogue 5′-*p*-fluorosulfonylbenzoylguanosine does not affect the intrinsic catalytic activity of the enzyme as measured in the absence of regulatory compounds, but leads to a progressive decrease in the sensitivity to inhibition by GTP. The modified enzyme binds only 1 mol of GTP per peptide chain, in the presence or absence of DPNH, implying that reaction with 5′-*p*-fluorosulfonylbenzoylguanosine eliminates one of the allosteric sites for GTP. In contrast, the Michaelis constants for substrates and the ability of the enzyme to be inhibited by high concentrations of DPNH are not appreciably changed by the modification reaction. Although the affinity for the activator ADP is not altered, the maximum extent of activation is decreased. The rate constant for reaction of glutamate dehydrogenase with 5′-*p*-fluorosulfonylbenzoylguanosine has been measured from the time dependence of the decreased inhibition by 1.1 μM GTP; this rate constant is specifically and strikingly decreased by low concentrations of GTP in the presence of reduced coenzyme but not by substrates, DPNH alone or ADP either with or without DPNH. The extent of covalent incorporation of radioactive 5′-sulfonylbenzoylguanosine is directly proportional to the percent decrease in GTP inhibition, a maximum alteration in sensitivity to GTP being observed when approximately 2 mol of 5′-sulfonylbenzoylguanosine is incorporated per enzyme subunit. Only 1 mol of reagent per peptide chain is covalently bound in the presence of GTP and reduced coenzyme, which protect the enzyme against the decreased response to GTP but do not prevent the decreased activation by ADP. In contrast, about 2 mol of reagent per enzyme subunit is incorporated in the presence of ADP and DPNH, which protect neither against the reduced response to ADP nor to GTP. These results suggest that incorporation of 1 mol of 5′-*p*-sulfonylbenzoylguanosine specifically causes elimination of one of the GTP sites of the native enzyme and that this change is responsible for the decreased sensitivity to GTP inhibition. Incorporation of the second mole of 5′-*p*-sulfonylbenzoylguanosine may occur at a site distinct from the recognized allosteric sites and indirectly cause a change in the extent of activation by ADP.

Bovine liver glutamate dehydrogenase (L-glutamate: $NAD(P)^+$ oxidoreductase (deaminating), EC 1.4.1.3) is an allosteric enzyme the activity of which is influenced by a variety of compounds which bind to distinguishable but mutually interacting sites. Notable among these compounds are GTP which inhibits, ADP which activates, and DPNH which, at relatively high concentrations, inhibits by binding to a site distinct from the catalytic site (Goldin & Frieden,

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1972a; Fisher, 1973). Although the adenosine and guanosine nucleotides appear to compete kinetically, chemical modification studies have indicated that the sites which they occupy are overlapping rather than identical (Colman & Frieden, 1966a; diPrisco, 1971). Similarly, although ADP may compete with high concentrations of DPNH for binding to the nonactive coenzyme site (Pantaloni & Dessen, 1969; Cross & Fisher, 1970; Koberstein et al., 1973; Pantaloni & Lecuyer, 1973), chemical desensitization of the enzyme toward DPNH inhibition has been accomplished without affecting its activation by ADP (Pal et al., 1975a).

Several chemical modification studies have aimed at elucidating the amino acid residues in the substrate or regulatory sites of glutamate dehydrogenase; however, the interpretation of many of these reports has been complicated either by the modification of multiple amino acid residues by a nonspecific reagent and/or by the concomitant alteration of several of the catalytic and allosteric properties of the enzyme. For example, reaction with bromopyruvate (Baker & Rabin, 1969), pyridoxal phosphate (Goldin & Frieden, 1972b), tetranitromethane (Price & Radda, 1969; Smith et al., 1970), and iodoacetamidosalicylic acid (Malcolm & Radda, 1970; Rosen et al., 1973) produce inactivation as well as decreased sensitivity to GTP inhibition; and reaction with trinitrobenzenesulfonate (Coffee et al., 1971; Goldin & Frieden, 1971) causes loss of DPNH inhibition in addition to an increase in the kinetically observed dissociation constant for GTP.

Affinity labeling using purine nucleotide analogues may provide the specificity for separately modifying the distinct regulatory sites of glutamate dehydrogenase. We have used this approach in examining the reaction of 5′-*p*-fluorosulfonylbenzoyladenosine with glutamate dehydrogenase (Pal et al., 1975a). In that case, reaction appears to occur specifically at the regulatory DPNH site since, when 1.1 mol of 5′-sulfonylbenzoyladenosine is incorporated per peptide chain, the resultant enzyme loses its ability to be inhibited by DPNH, but retains full activity as measured in the absence of allosteric ligands, is activated normally by ADP and is still inhibited 93% by GTP. In an attempt to label the allosteric GTP site of glutamate dehydrogenase, this investigation examines the reaction of 5′-*p*-fluorosulfonylbenzoylguanosine, an analogue of GTP which in addition to the guanosine moiety has a carbonyl group structurally similar to the α-phosphoryl group and a sulfonyl group analogous to the γ-phosphoryl group. The sulfonyl fluoride is a reactive functional group which is subject to nucleophilic attack by several amino acid side chains (Poulos & Price, 1974); therefore, specific covalent modification of the protein may occur directed by interaction of the reagent at a guanosine nucleotide site.

Glutamate dehydrogenase has been considered to have one binding site per peptide chain for GTP (Colman & Frieden, 1966b). This investigation extends the GTP concentration range over which the direct binding experiments are conducted and demonstrates that in the presence of the coenzyme DPNH there are two GTP binding sites per subunit which differ markedly in affinity for the nucleotide. Evidence is here presented indicating that 5′-*p*-fluorosulfonylbenzoylguanosine reacts at one of these sites.

Experimental Procedures

Materials. Bovine liver glutamate dehydrogenase, purchased from Boehringer Mannheim Corp. as a crystalline suspension in ammonium sulfate, was dialyzed in 40-mg batches for 16 h at 4 °C against two changes of 0.1 M potassium phosphate buffers, pH 7.1. The dialyzed material was then centrifuged at 4 °C for 30 min at 20 000 rpm to remove the precipitated denatured protein. The A_{280}/A_{260} ratio was 1.9 and the enzyme concentration was determined using the value $E_{279}^{1\%} = 9.7$ (Olson & Anfinsen, 1952). The enzyme was stored in frozen aliquots at −85 °C. For all calculations, a molecular weight of 56 100 for the identical peptide chains was used (Smith et al., 1970).

The compound 5′-*p*-fluorosulfonylbenzoylguanosine was prepared by reaction of guanosine hydrochloride with *p*-fluorosulfonylbenzoyl chloride as described previously (Pal et al., 1978). The [5′-^{3}H]-5′-*p*-fluorosulfonylbenzoylguanosine (0.0142 mCi/mol) was synthesized from [5′-^{3}H]guanosine purchased from New England Nuclear Corp. [U-^{14}C]GTP was also purchased from New England Nuclear Corp. All coenzymes and purine nucleotides, as well as EDTA and Tris base, were supplied by Sigma Chemical Co. Both DPNH and TPNH solutions were prepared immediately before use.

Enzymatic Assay. Enzymatic activity was measured spectrophotometrically at 340 nm from the oxidation of reduced coenzyme at 25 °C in Tris −0.01 M acetate buffer, pH 8 (containing 10 μM EDTA), using a Gilford Model 240 spectrophotometer equipped with an expanded scale recorder. For the standard assay the substrate concentrations used were 5 mM α-ketoglutarate, 50 mM ammonium chloride, and 100 μM DPNH or TPNH in a total volume of 1.0 mL. When the activity was measured in the presence of the inhibitor GTP, the nucleotide concentration was 1.1 μM. The Michaelis constants were also measured at pH 8 by maintaining the concentration of two substrates constant at the level of standard assay and varying the concentration of the third.

Reaction of Bovine Glutamate Dehydrogenase with 5′-p-Fluorosulfonylbenzoylguanosine. Glutamate dehydrogenase (0.4–0.8 mg/mL) was incubated with 5′-FSO_2BzGuo[1] (0.48–2.4 mM) at 25 °C in 0.01 M sodium barbital buffer (pH 8) containing 0.4 M KCl and 6 or 10% dimethylformamide. Dimethylformamide was necessary to maintain the solubility of 5′-FSO_2BzGuo in the reaction mixture and up to 10% dimethylformamide had no effect on the stability or on the measured activity of the enzyme. When rate constants were determined from changes in the kinetic parameters of the enzymes, aliquots were withdrawn at given time intervals, diluted 20-fold with Tris–0.1 M acetate buffer (pH 8) at 0 °C and assayed in the absence and presence of GTP. The overall dilution of the reaction mixture in the assay solution was about 600-fold.

Preparation of the Modified Enzymes. Glutamate dehydrogenase was incubated with 1.8 mM [5′-^{3}H]-5′-FSO_2BzGuo under the same conditions described above. During the course of the reaction, samples were withdrawn at various times and centrifuged for 3 min at 16 000 rpm and 4 °C to remove any precipitate. Excess reagent was removed by applying immediately the clear supernatant to a column of Sephadex G-25 (fine) (1 × 40 cm) equilibrated with 0.1 M potassium phosphate buffer, pH 7.1. The fractions containing glutamate dehydrogenase, detected by enzymatic assay, were pooled and the protein concentration was determined by reaction with the Bio-Rad Protein Assay reagent using native glutamate dehydrogenase to establish a standard curve. The modified enzymes were stored at −85 °C in frozen aliquots. "Protected enzyme" was prepared under similar conditions with a reaction mixture containing, in addition, GTP (0.1 mM) and TPNH (0.01 mM). The number of moles of 5′-sulfo-

[1] Abbreviations used: 5′-FSO_2BzGuo, 5′-*p*-fluorosulfonylbenzoylguanosine; 5′-SO_2BzGuo, 5′-*p*-sulfonylbenzoylguanosine; DPNH and TPNH, reduced diphosphopyridine and reduced triphosphopyridine nucleotides, respectively.

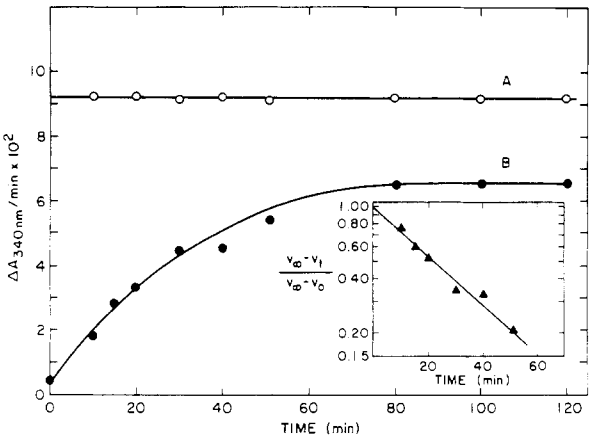

FIGURE 1: Reaction of 5-*p*-fluorosulfonylbenzoylguanosine with glutamate dehydrogenase. The enzyme (0.4 mg/mL) was incubated with 5′-FSO_2BzGuo (2.4 mM) at 25 °C in 0.01 M sodium barbital buffer (pH 8) containing 0.4 M KCl and 6% dimethylformamide. At each indicated time, a 30-μL aliquot was withdrawn, diluted 20-fold with Tris–0.1 M acetate buffer, and assayed (A) in the absence and (B) in the presence of 1.1 μM GTP and 100 μM DPNH as described in Experimental Procedures. (Inset) Determination of the pseudo-first-order rate constant from the increase in the observed velocity measured in the presence of GTP. V_t and V_0 are the enzymatic velocities measured with added GTP at the given time and zero time, respectively, and V_∞ is the constant velocity at the end of the reaction. The pseudo-first-order rate constant calculated is 0.032 min^{-1}.

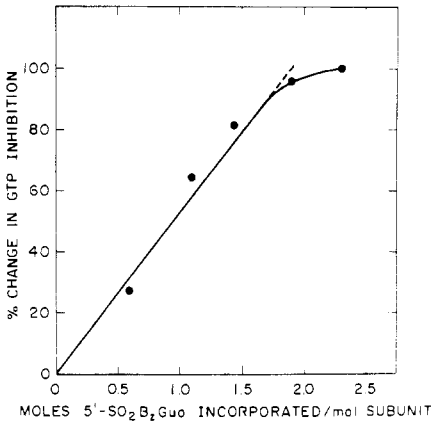

FIGURE 2: Percent change in GTP inhibition as a function of incorporation of 5′-SO_2BzGuo. Glutamate dehydrogenase (0.4 mg/mL) was incubated with radioactive 5′-FSO_2BzGuo (1.8 mM); aliquots were withdrawn at various times and assayed in the presence of 1.1 μM GTP. The excess reagent was removed by gel filtration as described in Experimental Procedures. The percent change in GTP inhibition is defined by $(V - V_0/V_\infty - V_0) \times 100$, where V is the enzymatic velocity measured at a given time with 1.1 μM GTP present, and V_0 and V_∞ are the velocities measured under the same conditions at zero time and at the end of the reaction, respectively.

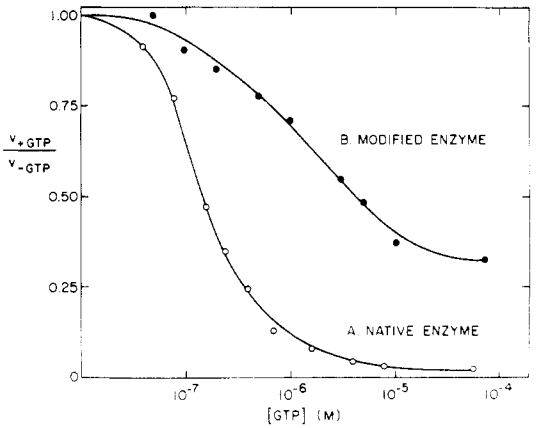

FIGURE 3: Ratio of maximum velocities in the presence and absence of GTP for native and modified enzymes. The velocities were measured at 100 μM DPNH with (A) native and (B) modified enzyme containing 2.3 mol of 5′-SO_2BzGuo incorporated per subunit.

nylbenzoylguanosine incorporated was determined by measuring the radioactivity in ACS scintillation liquid (Amersham) using a Packard Model 3300 liquid scintillation counter.

GTP Binding Studies. Binding of [U-^{14}C]GTP to native and modified enzymes was measured by the ultrafiltration technique (Colman & Foster, 1970) at 25 °C in Tris–0.05 M acetate buffer (pH 7.1) containing 10 mM potassium phosphate and 100 μM EDTA. An Amicon Model 10-PA ultrafiltration cell was assembled with a PM-10 membrane to separate the free ligand from enzyme-bound ligand. In the absence of enzyme, retention of radioactivity by the membrane was less than 1%. Both free and total ligand concentrations were measured from the radioactivity using a liquid scintillation counter. The concentration of bound ligand was determined from the difference between the concentrations of total and free ligand.

Results

Reaction of Glutamate Dehydrogenase with 5′-p-Fluorosulfonylbenzoylguanosine. Incubation of glutamate dehydrogenase with 2.4 mM 5′-FSO_2BzGuo for 2 h produces no change in the maximum velocity when examined without modifiers (Figure 1, curve A). This result implies that 5′-FSO_2BzGuo does not react at the active site. When assayed in the presence of 1.1 μM GTP, native glutamate dehydrogenase exhibits a maximum velocity that is only 6% as compared with the maximum velocity measured in the absence of modifier. In contrast to its lack of effect on the intrinsic catalytic activity of the enzyme, 5′-FSO_2BzGuo produces a progressive decrease in the ability of the enzyme to be inhibited by GTP, as indicated by an increase in the velocity measured in the presence of a constant concentration of GTP (Figure 1, curve B). After 80-min incubation, the velocity measured in the presence of 1.1 μM GTP is increased to 72% of that in the absence of the modifier. This value remains constant upon continuation of the incubation period. (These data were obtained using 100 μM DPNH in the enzyme assay; however, similar results were obtained when 100 μM TPNH was used for the assay.)

The guanosine analogue [5′-H]-5′-FSO_2BzGuo reacts covalently with bovine liver glutamate dehydrogenase, as demonstrated by the retention of radioactivity in the enzyme peak after gel filtration on Sephadex G-25. The incorporation of radioactive reagent increases progressively with the time of incubation of 1.8 mM 5′-FSO_2BzGuo with enzyme; after 180 min, 2.3 mol of reagent is incorporated per peptide chain. Figure 2 shows that the percent change in GTP inhibition is directly proportional to the extent of incorporation of 5′-SO_2BzGuo. The maximum change in sensitivity to GTP inhibition is observed when approximately 2 mol of guanosine analogue per mol of subunit are incorporated.

Kinetic Properties of Modified Enzyme. In order to elucidate the effect of this limited modification of the enzyme, the catalytic and regulatory characteristics of enzyme containing 2.3 mol of covalently bound 5′-SO_2BzGuo per peptide chain were examined in comparison with those of native glutamate dehydrogenase. The data of Figure 1 indicated that reaction with 5′-FSO_2BzGuo led to a decrease in, but not an elimination of, the inhibitory effect of GTP on the enzyme. As a means of ascertaining whether there is any alteration in the affinity of the enzyme for GTP, the velocities of native and modified enzymes are compared in Figure 3 as a function

Table I: Michaelis Constants for Native and Modified Enzyme

enzyme	$K_{\alpha\text{-ketoglutarate}}$ (mM)	$K_{NH_4^+}$ (mM)	$K_{DPNH\text{-}1}$ (μM)	K_{TPNH} (μM)
native	0.90	7.7	66	33
modified[a]	0.87	7.7	83	50

[a] Modified enzyme contained 2.3 mol of 5′-SO_2BzGuo incorporated per subunit.

of GTP concentration. The dissociation constant has been shown to be numerically equal to the concentration of GTP at which the maximum velocity is described by

$$1/2(V_0 + V_I) \quad (1)$$

where V_0 and V_I are the maximum velocities in the absence and presence, respectively, of saturating concentrations of GTP (Frieden, 1963). Figure 3, curve B, shows that the dissociation constant of the GTP-modified enzyme complex is 1.6 μM, ten times higher than the constant of 0.14 μM measured for native glutamate dehydrogenase from Figure 3, curve A. At saturating concentrations of GTP, the maximum velocity for native enzyme is reduced to 2% of that observed in the absence of the inhibitor; however, for modified enzyme, the maximum velocity is decreased only to 32% of that in the absence of GTP.

GDP is known to inhibit glutamate dehydrogenase by binding, albeit more weakly than GTP, to an allosteric site (Frieden, 1963). As in the case of GTP, the inhibition by GDP is significantly altered by reaction of the enzyme with 5′-FSO_2BzGuo. The kinetically measured dissociation constants for enzyme–GDP complex are 6.4 and 50 μM for the native and modified enzymes, respectively. At saturating levels of GDP, the maximum velocity is decreased to 5% for native, as compared with 43% for the fully modified enzyme.

In addition to affecting the ability of glutamate dehydrogenase to be inhibited by GTP and GDP, reaction with 5′-FSO_2BzGuo also alters its response to ADP. The dissociation constant for the enzyme–ADP complex is numerically equal to the concentration of ADP at which the maximum velocity is given by

$$1/2(V_0 + V_A) \quad (2)$$

where V_0 and V_A are the maximum velocities in the absence and presence, respectively, of saturating concentrations of ADP (Frieden, 1963). The concentration dependence for activation of native and modified enzyme by ADP, shown in Figure 4, reveals that both enzymes have a similar affinity for ADP, although the extent of activation is significantly decreased as a result of the modification reaction. The dissociation constant of 19 μM for the modified enzyme is comparable to 16 μM for the native enzyme; however, the modified enzyme is activated only 1.5-fold, whereas the maximum velocity of the native enzyme is 2.8-fold.

High concentrations of DPNH are known to inhibit glutamate dehydrogenase by binding to a second site distinct from the catalytic site as well as from the allosteric sites occupied by GTP and ADP (Frieden, 1959, 1963). As shown in Figure 5, although they may differ slightly in affinity for coenzyme, both the native and modified enzymes are inhibited by high concentrations of DPNH. These results indicate that the DPNH regulatory site is not the primary site of attack of 5′-FSO_2BzGuo.

Similarly, as recorded in Table I, the Michaelis constants for the substrates α-ketoglutarate and ammonium ion are not affected by modification of the enzyme by 5′-*p*-fluorosulfonylbenzoylguanosine, and the K_m for TPNH is only minimally increased. A Michaelis constant of 83 μM is

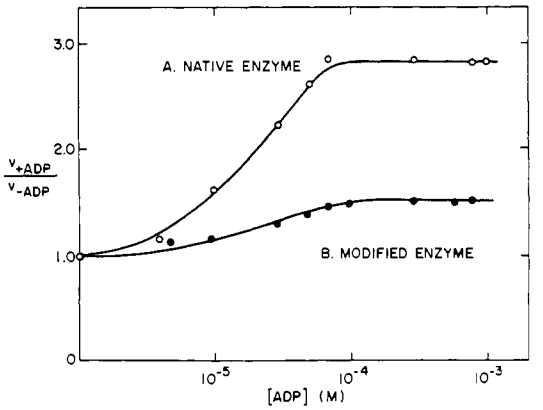

FIGURE 4: Ratio of maximum velocities in the presence and absence of ADP as a function of ADP concentration for native and modified glutamate dehydrogenase. (A) Native and (B) modified enzyme containing 2.3 mol of 5′-SO_2BzGuo incorporated per peptide chain. The velocities were measured under the conditions described in Experimental Procedures, using 100 μM DPNH as coenzyme.

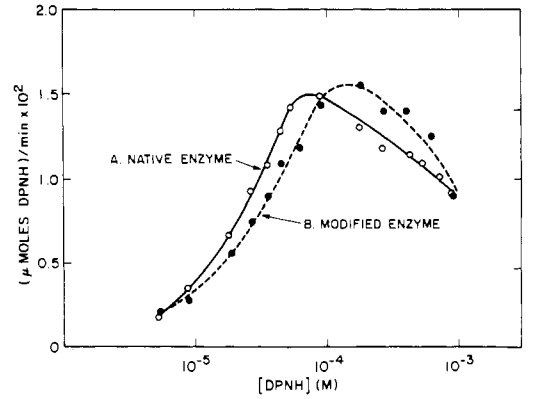

FIGURE 5: Initial velocity as a function of DPNH concentration for native and modified enzymes. (A) Native enzyme and (B) modified enzyme with 2.3 mol of 5′-SO_2BzGuo incorporated per peptide chain. At DPNH concentrations up to 2×10^{-4} M, velocity measurements were made at 340 nm as described in Experimental Procedures. Measurements at higher concentrations were conducted at 375 nm. The value of $\epsilon^{375} = 1.85 \times 10^3$ M^{-1} cm^{-1} for DPNH was used in calculating the rate in units of μmol of DPNH/min.

obtained from the linear portion of the double-reciprocal plot of $1/v$ vs. $1/(DPNH)$ for the modified enzyme, a value only slightly higher than the 66 μM measured for the native enzyme.

Kinetics of Reaction of Glutamate Dehydrogenase with 5′-FSO_2BzGuo. The most profound difference between the kinetic characteristics of the modified and native glutamate dehydrogenase is the altered affinity for GTP, as exemplified by Figure 3. The change from curve A to curve B during the course of incubation of enzyme with 5′-FSO_2BzGuo can be monitored by assaying the enzyme in the presence of a constant concentration of GTP, such as 10^{-6} M. This approach provides a convenient method for determining the rate constant for the reaction of the guanosine analogue with glutamate dehydrogenase at the site involving GTP inhibition. As illustrated in the inset of Figure 1, the change in enzymatic velocity

Table II: Rate of Reaction of 5′-*p*-Fluorosulfonylbenzoylguanosine with Glutamate Dehydrogenase in the Presence of Substrates and Modifiers[a]

addition to reaction mixture	$k_{obsd} \times 10^3$ (min^{-1})
1. none	23.3
2. α-ketoglutarate (20 mM)	17.3
3. TPNH (0.1 mM)	18.2
4. DPNH (0.1 mM)	22.7
5. TPNH (3.3 mM)	6.6
6. DPNH (3.0 mM)	16.7
7. ADP (2.0 mM)	18.2
8. ADP (2.0 mM) + DPNH (0.1 mM)	19.6
9. ADP (2.0 mM) + DPNH (0.1 mM) + α-ketoglutarate (20 mM)	14.7
10. GTP (10.0 μM)	10.9
11. GTP (100 μM)	10.1
12. GTP (1.0 μM) + DPNH (0.1 mM)	8.1
13. GTP (2.0 μM) + DPNH (0.1 mM)	7.9
14. GTP (10.0 μM) + DPNH (0.1 mM)	3.3
15. GTP (100 μM) + DPNH (0.1 mM)	0.7
16. GTP (10.0 μM) + TPNH (0.1 mM)	1.6
17. GTP (100 μM) + TPNH (0.1 mM)	0.4
18. GDP (100 μM) + DPNH (0.1 mM)	12.4
19. GDP (3.0 mM) + DPNH (0.1 mM)	1.4

[a] Glutamate dehydrogenase (0.4 mg/mL) was incubated with 1.9 mM 5′-FSO_2BzGuo at 25 °C in 0.01 M sodium barbital buffer (pH 8.0) and the ionic strength was adjusted to 0.4 M by the addition of KCl. The pseudo-first-order rate constants for the reaction were determined in accordance with the inset of Figure 1.

observed in the presence of 1.1 μM GTP obeys pseudo-first-order kinetics, yielding in that case a rate constant of 0.032 min^{-1} for reaction with 2.4 mM 5′-FSO_2BzGuo.

The pseudo-first-order rate constant for modification of glutamate dehydrogenase was measured as a function of 5′-FSO_2BzGuo concentration over the range 0.48–2.4 mM. A plot of the observed rate constant as a function of the concentration of 5′-FSO_2BzGuo is linear, indicating that a rapidly reversible complex of enzyme and reagent is not formed prior to the irreversible modification. A second-order rate constant of 13 min^{-1} M^{-1} may be calculated from the slope of the line. The guanosine moiety is an important determinant of the reaction of 5′-FSO_2BzGuo with glutamate dehydrogenase. Incubation of the enzyme with *p*-fluorosulfonylbenzoic acid, a compound which has the same functional group as 5′-FSO_2BzGuo but which lacks the guanosine moiety, produces only a very slow decrease in the sensitivity of the enzyme to GTP inhibition; at 2.0 mM, the observed rate constant is 0.0012 min^{-1} for *p*-fluorosulfonylbenzoic acid, which is less than 5% of the rate constant obtained for the same concentration of 5′-FSO_2BzGuo (0.0265 min^{-1}).

The effect of added substrates and regulatory compounds on the observed rate constant for modification of glutamate dehydrogenase by 1.9 mM 5′-FSO_2BzGuo is recorded in Table II. Little decrease in the rate constant is caused by the substrate α-ketoglutarate or by the coenzyme DPNH or TPNH (either alone or in combination with substrate) when present at concentrations sufficient to bind to the catalytic site (lines 2–4 and 9). These results are consistent with a postulate that the locus of modification by 5′-FSO_2BzGuo is not at the active site. At the high concentrations of reduced coenzyme at which the second sites would be saturated (Krause et al., 1974), DPNH does not appreciably reduce the rate constant and TPNH causes only partial protection against modification (lines 5 and 6). Similarly, the allosteric activator ADP, whether present alone or together with DPNH, causes only a slight decrease in the rate constant (lines 7 and 8), implying that the changes in the kinetic characteristics of the enzyme produced by reaction with 5′-FSO_2BzGuo do not result directly from modification of the ADP site.

The rate constant is decreased only twofold by the addition to the reaction mixture of the allosteric inhibitor GTP by itself (lines 10 and 11); in contrast, there is a striking decrease in k_{obsd} when GTP is added together with reduced coenzyme (lines 12–17). GTP is known to bind more tightly to glutamate dehydrogenase in the presence of the coenzyme DPNH or TPNH (Colman & Frieden, 1966b), and indeed the response of the enzyme to GTP is so different in the absence and presence of coenzyme as to raise the question of whether the same sites are occupied by GTP under both conditions. Table II shows that, in the presence of reduced coenzyme, up to a 14-fold decrease in the rate constant is produced by GTP concentrations in the range 1–10 μM, and almost complete protection is provided by 100 μM GTP. Similarly, the presence of 3.0 mM GDP in combination with DPNH produces striking protection against the kinetic effects of modification. These results suggest that 5′-FSO_2BzGuo normally reacts in the region of a GTP–GDP binding site.

Properties of Enzyme Modified in the Presence of GTP or ADP and Reduced Coenzyme. Incubation of radioactive 1.8 mM 5′-FSO_2BzGuo with glutamate dehyrogenase for 180 min in the presence of 0.1 mM GTP and 0.1 mM TPNH yields enzyme which exhibits no change in activity when assayed with 1.1 μM GTP. This "protected enzyme" contains only 1.0 mol of 5′-SO_2BzGuo per subunit, as compared with 2.3 mol per subunit observed when enzyme is incubated under the same conditions but in the absence of nucleotides. These results suggest that the extra mole of reagent which is incorporated in the absence of GTP and TPNH is responsible for the diminished inhibition by the allosteric modifier GTP.

The "protected enzyme" exhibits a dependence of inhibition on GTP concentration which is similar to that of native enzyme. The dissociation constant for the GTP–"protected enzyme" complex is 0.22 μM, as compared with 0.14 μM for native enzyme; and the maximum velocities at saturating concentrations of GTP are 4% and 2% of that in the absence of GTP for "protected" and native enzymes, respectively. However, this "protected enzyme" still exhibits a diminished activation by ADP; the maximum extent of activation is 1.7-fold as compared with 2.8-fold for native enzyme, although K_{ADP} is the same as that of native enzyme (16 μM). Since protection against loss of GTP inhibition does not confer protection against the change in response to ADP, it appears that these two effects result from modification of different amino acids. The 1 mol of 5′-SO_2BzGuo which is still incorporated in the presence of GTP and DPNH may be responsible for the decreased activation by ADP.

In contrast to the effect of GTP, incubation of glutamate dehydrogenase with 1.8 mM radioactive 5′-FSO_2BzGuo for 180 min in the presence of 2 mM ADP and 0.1 mM DPNH yields enzyme with 2.4 mol of 5′-SO_2BzGuo incorporated per peptide chain, a value not significantly different from that observed in the absence of nucleotides. This enzyme exhibits essentially the same loss in sensitivity to GTP inhibition as does enzyme incubated in the absence of ADP and DPNH: the maximum velocity at saturating concentrations of GTP is 32% of that in the absence of GTP and K_{GTP} is 1.5 μM. Furthermore, this enzyme exhibits an altered response when assayed with ADP. It has a K_{ADP} of 23 μM and a maximum extent of activation of only 1.7-fold. The failure of ADP and DPNH to protect against either the change in response to the

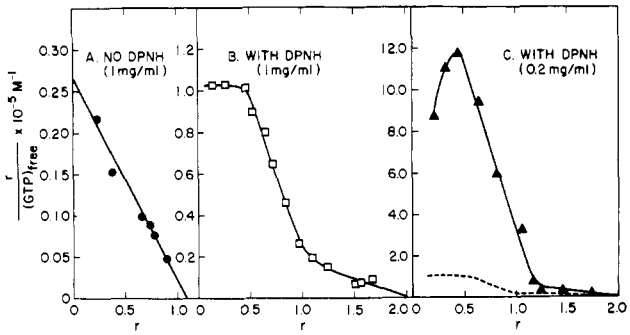

FIGURE 6: Binding of GTP to native glutamate dehydrogenase in Tris–0.05 M acetate buffer (pH 7.1) containing 10 mM potassium phosphate and 100 μM EDTA. A and B record data measured in the absence and in the presence of 0.1 mM DPNH, respectively, at the enzyme concentration of 1.0 mg/mL. (C) The solid line with experimental points records data for 0.2 mg/mL of glutamate dehydrogenase in the presence of 0.1 mM DPNH. The dotted line reproduces the curve from B, obtained at 1.0 mg/mL, to facilitate direct comparison between the two protein concentrations.

inhibitor GTP or to the activator ADP upon reaction of the enzyme with 5′-FSO_2BzGuo suggests that neither change is a direct result of modification of the ADP site. It is likely that the change in the extent of activation by ADP is an indirect result of modification of a site distinct from the recognized allosteric sites.

Binding of GTP by Native and Modified Glutamate Dehydrogenase. Although the protection by 0.1 mM GTP and reduced coenzyme against the modification of glutamate dehydrogenase by 5′-*p*-fluorosulfonylbenzoylguanosine strongly suggests that 5′-SO_2BzGuo reacts at the allosteric GTP site, the modified enzyme is still inhibited by GTP as observed in kinetic experiments (Figure 3). In order to evaluate whether 5′-SO_2BzGuo reacts at a GTP site of the enzyme, binding of [U-^{14}C]GTP to native and modified glutamate dehydrogenase was studied using an ultrafiltration technique. The binding data were analyzed in accordance with the Scatchard equation

$$\frac{r}{(\mathrm{GTP})_{\mathrm{free}}} = \frac{n}{K} - \frac{r}{K} \qquad (3)$$

where r is the number of moles of GTP bound per mole of peptide chain; n is the number of GTP binding sites per subunit; and K is the dissociation constant for the enzyme–GTP complex. These measurements were made in Tris–0.05 M acetate, pH 7.1, containing 10 mM potassium phosphate to maintain the stability of the enzyme. In the absence of DPNH, the binding of GTP to native glutamate dehydrogenase exhibits a linear Scatchard plot (Figure 6A). These data yield a value of 1.10 for the number of GTP sites per peptide chain, with a dissociation constant of 42 μM. In contrast, there are two binding sites for GTP per peptide chain in the presence of 0.1 mM DPNH (Figure 6B). Binding to the first site exhibits a distinct nonlinearity, which is noted particularly in the region of $r < 0.5$; this observation is suggestive of an apparent cooperativity among the subunits, as has been noted previously (Colman & Frieden, 1966b). The dissociation constant as obtained from the limiting slope is 4.7 μM for the first site. Binding to the second site is considerably weaker, as indicated by an estimated dissociation constant of 44 μM. For comparison, a dissociation constant of only 0.3 μM is measured kinetically from the inhibition by GTP of native glutamate dehydrogenase under similar conditions at pH 7.1; the kinetically determined value is thus considerably lower than the constant for even the high affinity GTP site obtained from the ultrafiltration experiments. It may be

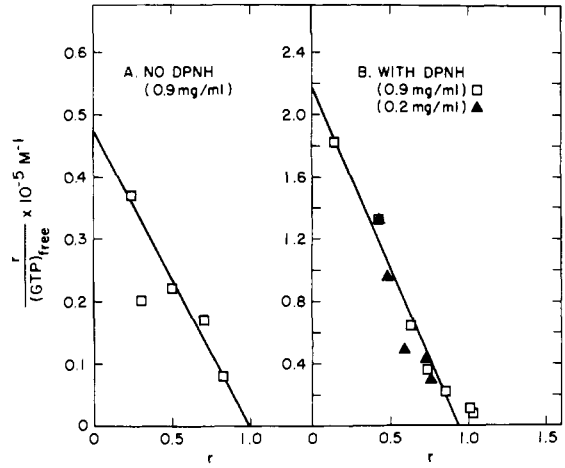

FIGURE 7: Binding of GTP to modified glutamate dehydrogenase containing 2.3 mol of 5′-SO_2BzGuo incorporated per subunit in Tris–0.05 M acetate buffer (pH 7.1) containing 10 mM potassium phosphate and 100 μM EDTA. A records data for enzyme in the absence of DPNH at an enzyme concentration of 0.9 mg/mL, and B shows data for the enzyme concentrations of 0.9 and 0.2 mg/mL measured in the presence of 0.1 mM DPNH.

relevant that in the kinetic measurements the concentration of enzyme is several orders of magnitude lower than the 1 mg/mL used for these ultrafiltration experiments. Frieden & Colman (1967) reported earlier that binding of GTP is dependent on the state of polymerization of the enzyme; thus the dissociation constant decreases as the concentration and state of aggregation of glutamate dehydrogenase are decreased. The Scatchard plot for binding of GTP to 0.2 mg/mL of glutamate dehydrogenase (Figure 6C) yields a dissociation constant of 0.61 μM from the limiting slope for the high affinity site. The reduction in protein concentration thus leads to a decrease of more than sevenfold in the dissociation constant for the tight site to a level which is much closer to the 0.3 μM determined kinetically at very low protein concentrations. The dissociation constant for the low affinity site is affected to a lesser extent by the decrease in protein concentration: K is estimated at 18 μM for this second site, a decrease of only twofold as compared with that measured at 1 mg/mL.

In the absence of DPNH, modified enzyme with 2.3 mol of 5′-SO_2BzGuo bound per peptide chain binds 1.0 mol of GTP per subunit (Figure 7A), with a dissociation constant (K = 23.8 μM) not very different from that of native enzyme. A major distinction is observed in the presence of DPNH between the binding of GTP by modified (Figure 7B) and native enzyme (Figures 6B and 6C). Only 1 mol of GTP is bound by the modified enzyme (even at high concentrations of the guanosine nucleotide), with a dissociation constant of 4.5 μM. Therefore, one binding site for GTP is eliminated as a result of the modification of glutamate dehydrogenase by 5′-*p*-fluorosulfonylbenzoylguanosine. A linear Scatchard plot is observed for modified enzyme in the presence of DPNH; thus, there is no evidence for apparent cooperativity among the subunits for binding of the 1 mol of GTP/subunit. Moreover, the dissociation constant for GTP is not dependent on the concentration of the modified enzyme: the experimental points obtained at 0.2 and 0.9 mg/mL are colinear (Figure 7B), in contrast to the results obtained for native enzyme (Figure 6C).

It is notable that the modified enzyme retains the ability to polymerize under the conditions used for the GTP binding experiments (i.e., in Tris–0.05 M acetate buffer, pH 7.1, containing 10 mM potassium phosphate, 100 μM EDTA, and 100 μM DPNH); the weight average molecular weight as

determined by light scattering measurements at a protein concentration of 0.42 mg/mL is 1 560 000. (The completely, but reversibly, disaggregated species has a molecular weight of 336 000.) Upon addition of 100 μM GTP, the measured molecular weight is 1 510 000, indicating that the combination of GTP and DPNH does *not* cause dissociation of this modified enzyme. In contrast, weight average molecular weights of 1 840 000 and 774 000 are measured for native enzyme at 0.42 mg/mL in the same buffer in the presence of 100 μM DPNH alone and of 100 μM GTP plus 100 μM DPNH, respectively; these results are in agreement with the known ability of GTP and DPNH to promote dissociation of the native enzyme (Colman & Frieden, 1966b).

Discussion

The compound 5′-*p*-fluorosulfonylbenzoylguanosine was designed as an affinity label for guanosine nucleotide sites in proteins. Thus, before evaluating the effect of the reagent on the properties of glutamate dehydrogenase, it is necessary to clarify the number and nature of the GTP binding sites on the enzyme. The data obtained in this investigation indicate that in the presence of DPNH the native enzyme has two binding sites for GTP per peptide chain with dissociation constants of about 0.6 and 18 μM, respectively. Colman & Frieden (1966b), on the basis of direct binding experiments, reported that glutamate dehydrogenase has one GTP binding site per peptide chain in the presence of DPNH. However, the highest concentration of GTP used in that study was only 8 μM, thus accounting for the fact that the weak binding site was overlooked. The high affinity site for GTP now observed seems to correspond to the single GTP site described earlier. The two studies differed in the concentration of inorganic phosphate present in the buffers: 1 mM was used in the previous investigation, whereas the binding experiments here reported were conducted in buffer containing 10 mM phosphate, in order to enhance the stability of the enzyme. Inorganic phosphate has been reported to weaken the binding of GTP to glutamate dehydrogenase (Frieden, 1976; Koberstein et al., 1977) and therefore it is not unexpected that the dissociation constants for GTP would be about twofold higher in the current study. Nevertheless, the characteristics of the high affinity site for GTP shown in Figure 6 are comparable to those observed previously for GTP in the presence of reduced coenzyme: there is an apparent cooperativity among the subunits for the binding of the nucleotide to the tighter site which is not noted for binding to the low affinity site. Furthermore, the state of polymerization of the enzyme has been shown to play an important role in the binding of GTP in the presence of DPNH (Frieden & Colman, 1967); a decrease in the enzyme concentration from 1 to 0.2 mg/mL is here found to cause a sevenfold decrease in the dissociation constant for GTP from the high affinity site but only about a twofold decrease in the dissociation constant for the low affinity site. In contrast to these results, in the absence of coenzyme, glutamate dehydrogenase exhibits only one site for GTP per peptide chain, which is characterized by a linear Scatchard plot and a dissociation constant comparable to that of the weaker site observed in the presence of DPNH. It is not possible to state with certainty whether these two weaker sites, observed in the absence and presence of DPNH, are the same or different.

The existence of more than one GTP binding site per subunit has been indicated by several recent studies. Andree & Zantema (1978) reported on the basis of NMR studies that besides the high affinity site, glutamate dehydrogenase has another low affinity site for GTP per subunit. An additional kinetically inhibitory site at high GTP concentrations has been detected by Koberstein et al. (1977). Since there are at least two GTP sites per peptide chain, it might be anticipated that a GTP analogue could react at either one or more of these sites.

Binding studies conducted in the presence of DPNH for enzyme modified by 5′-FSO_2BzGuo demonstrate that one GTP site per peptide chain has been eliminated. Although it is not easy to ascertain definitively which of the two sites has been affected by the modification, the tighter site seems to be the most probable candidate. There is a lack of apparent cooperativity among the subunits for binding to the residual GTP site and, furthermore, the dissociation constant for GTP is not altered by a change in the concentration of modified enzyme, despite the fact that the modified enzyme retains the ability to polymerize. These characteristics are similar to those of the low affinity GTP site of the native enzyme. Furthermore, the addition of GTP and DPNH to the modified enzyme does not result in appreciable depolymerization, as would be expected for binding of GTP to the tight site (Frieden, 1963). However, the dissociation constant for GTP is significantly decreased in the presence of DPNH as observed in the case of the high affinity site of the native enzyme. The binding constant in the presence of DPNH (4.5 μM) is not too dissimilar from that measured kinetically for the modified enzyme (1.5–2 μM), implying that the partial inhibition by GTP of the modified enzyme results from binding to this residual GTP site.

The function of the region of glutamate dehydrogenase attacked by 5′-*p*-fluorosulfonylbenzoylguanosine can be further elucidated by considering both the kinetic parameters of the enzyme which are altered as a result of the covalent reaction, as well as the pattern of ligands which provide protection against the modification reaction. The catalytic site does not appear to be modified by 5′-FSO_2BzGuo, since incubation with the reagent does not alter the catalytic activity of the enzyme when measured at high substrate concentrations in the absence of the regulatory compounds. Moreover, in the modified enzyme the Michaelis constants for α-ketoglutarate, TPNH and low concentrations of DPNH are not appreciably different from those of the native enzyme. Furthermore, the substrates α-ketoglutarate and low concentrations of TPNH and DPNH do not provide protection against the modification, indicating that the catalytic site is not the locus of attack by 5′-FSO_2BzGuo.

The regulatory sites are then the most probable loci for modification by 5′-*p*-fluorosulfonylbenzoylguanosine. In contradistinction to the modification produced by the affinity labels 5′-*p*-fluorosulfonylbenzoyladenosine (Pal et al., 1975a) and 3′-*p*-fluorosulfonylbenzoyladenosine (Pal et al., 1975b), the region of attack by 5′-FSO_2BzGuo is not at the inhibitory DPNH site of the enzyme, since the rate constant for the reaction is not significantly affected by high concentrations of DPNH. Although the examination of the DPNH dependence of velocity shows a slight increase in K_m, the modified enzyme is still inhibited by high concentrations of DPNH (Figure 5).

No specific effect on the regulatory ADP site of the enzyme seems to result from modification of glutamate dehydrogenase by 5′-FSO_2BzGuo. Although there is a decrease in the extent of activation produced in the modified enzyme by high concentrations of ADP, the affinity of the altered enzyme for ADP is essentially the same as that of the native enzyme. Furthermore, the inclusion of ADP and DPNH in the reaction mixture does not prevent the reduction in the extent of activation produced by reaction of enzyme with 5′-FSO_2BzGuo, suggesting that this characteristic of the modified enzyme is

not caused by direct reaction within the ADP site.

The most striking effect of the reaction of 5′-FSO_2BzGuo with glutamate dehydrogenase is the decrease in sensitivity toward GTP inhibition: the maximum extent of inhibition is only 68% for modified as compared with 98% for native enzyme and the K_{GTP} is increased to 1.6 μM from 0.14 μM in the native enzyme. This modification can be substantially prevented by including in the incubation mixture DPNH together with GTP in a concentration range required for binding to the high affinity site (1–10 μM). If 5′-FSO_2BzGuo reacted at the low affinity GTP site, one might have expected that GTP concentrations of more than 40 μM would be required to produce a significant decrease in the rate constant for the modification reaction and that GTP would provide marked protection in the absence of reduced coenzyme; neither of these expectations is realized. These observations indicate that 5′-*p*-fluorosulfonylbenzoylguanosine reacts covalently at the high affinity GTP site of glutamate dehydrogenase thus preventing GTP from binding to that site and inhibiting the enzyme; the 5′-SO_2BzGuo moiety does not itself appear to inhibit the enzyme or to promote the disaggregation of polymeric forms of glutamate dehydrogenase. In the case of native enzyme, the 98% inhibition is probably produced by the binding of GTP to the high affinity site. When binding of the guanosine nucleotide to this site is eliminated by reaction with 5′-FSO_2BzGuo, the weaker inhibition resulting from binding of GTP to the low affinity site is revealed.

The reaction of 5′-*p*-fluorosulfonylbenzoylguanosine with glutamate dehydrogenase has many of the characteristics of affinity labeling. The guanosine portion of the structure plays an important role in the reaction, since *p*-fluorosulfonylbenzoic acid, having the same alkylating group but lacking the nucleoside, reacts very slowly as compared with the same concentration of 5′-FSO_2BzGuo. Moreover, the compound 5′-*p*-fluorosulfonylbenzoyladenosine, which has an adenosyl in place of the guanosyl moiety, reacts differently with glutamate dehydrogenase: it produces an altered enzyme which is still inhibited 93% by GTP and is not inhibited by high concentrations of DPNH. 5′-FSO_2BzGuo does not behave as a generalized alkylating agent but rather reacts at a limited number of sites: only about two moles of 5′-SO_2BzGuo are incorporated per enzyme subunit concomitant with the decrease in sensitivity to GTP inhibition and loss of one GTP binding site, as well as a decrease in the maximum extent of activation by ADP. The presence of GTP and reduced coenzyme in the reaction mixture specifically reduces the incorporation to 1 mol of 5′-SO_2BzGuo per enzyme subunit and prevents the change in response of the enzyme to GTP without affecting the decrease in activation by ADP. In contrast, the presence of an ADP and reduced coenzyme alters neither the extent of incorporation nor the response of the enzyme to ADP or GTP. Reaction of 1 mol of 5′-FSO_2BzGuo thus takes place at a specific GTP site, whereas reaction of the second mole of reagent appears to occur at a nonspecific site which indirectly causes a decrease in the extent of activation by ADP.

In contrast to the usual expectation for an affinity label, the reagent concentration dependence of the rate constant for the reaction of 5′-FSO_2BzGuo with glutamate dehydrogenase is linear, implying that no reversible binding takes place prior to the irreversible modification. Either the rate of formation of the enzyme 5′-SO_2BzGuo complex is relatively slow or there is no appreciable binding prior to the covalent modification.

The investigations of the effects on glutamate dehydrogenase of reaction with 5′-*p*-fluorosulfonylbenzoyladenosine (Pal et al., 1975a), with 3′-*p*-fluorosulfonylbenzoyladenosine (Pal et al., 1975b), and with 5′-*p*-fluorosulfonylbenzoylguanosine illustrate the importance of having available several nucleotide analogues to differentially map various purine nucleotide sites on proteins. It is anticipated that 5′-*p*-fluorosulfonylbenzoylguanosine will have broad applications for the labeling of the catalytic sites of GTP-dependent kinases as well as of allosteric GTP sites in a variety of proteins.

References

Andree, P. J., & Zantema, A. (1978) *Biochemistry 17*, 778.

Baker, J. P., & Rabin, B. R. (1969) *Eur. J. Biochem. 11*, 154.

Coffee, C. J., Bradshaw, R. A., Goldin, B. R., & Frieden, C. (1971) *Biochemistry 10*, 3516.

Colman, R. F., & Frieden, C. (1966a) *J. Biol. Chem. 241*, 3652, 3661.

Colman, R. F., & Frieden, C. (1966b) *Biochem. Biophys. Res. Commun. 22*, 100.

Colman, R. F., & Foster, D. S. (1970) *J. Biol. Chem. 245*, 6190.

Cross, D. G., & Fisher, H. F. (1970) *J. Biol. Chem. 245*, 2612.

diPrisco, G. (1971) *Biochemistry 10*, 585.

Fisher, H. F. (1973) *Adv. Enzymol. 39*, 369.

Frieden, C. (1959) *J. Biol. Chem. 234*, 809, 815.

Frieden, C. (1963) *J. Biol. Chem. 238*, 3286.

Frieden, C. (1976) *The Urea Cycle (Conference)* (Grisolia, S., Baguena, R., & Mayor, F., Eds.) p 59, Wiley, New York.

Frieden, C., & Colman, R. F. (1967) *J. Biol. Chem. 242*, 1705.

Goldin, B. R., & Frieden, C. (1971) *Biochemistry 10*, 3527.

Goldin, B. R., & Frieden, C. (1972a) *Curr. Top. Cell. Regul. 4*, 77.

Goldin, B. R., & Frieden, C. (1972b) *J. Biol. Chem. 247*, 2139.

Koberstein, R., Krause, J., & Sund, H. (1973) *Eur. J. Biochem. 40*, 543.

Koberstein, R., Dieter, H., Markau, K., & Sund, H. (1977) 2nd International Symposium on Pyridine Nucleotide Dependent Dehydrogenases, Konstanz.

Krause, J., Buhner, M., & Sund, H. (1974) *Eur. J. Biochem. 41*, 593.

Malcolm, A. D. B., & Radda, G. K. (1970) *Eur. J. Biochem. 15*, 555.

Olson, J. A., & Anfinsen, C. B. (1952) *J. Biol. Chem. 197*, 67.

Pal, P. K., Wechter, W. J., & Colman, R. F. (1975a) *J. Biol. Chem. 250*, 8140.

Pal, P. K., Wechter, W. J., & Colman, R. F. (1975b) *Biochemistry 14*, 707.

Pal, P. K., Reischer, R. J., Wechter, W. J., & Colman, R. F. (1978) *J. Biol. Chem. 254*, 6644.

Pantaloni, D., & Dessen, P. (1969) *Eur. J. Biochem. 11*, 510.

Pantaloni, D., & Lecuyer, B. (1973) *Eur. J. Biochem. 40*, 381.

Poulos, T. L., & Price, P. A. (1974) *J. Biol. Chem. 249*, 1453.

Price, N. C., & Radda, G. K. (1969) *Biochem. J. 114*, 419.

Rosen, N. L., Bishop, L., Burnett, J. B., Bishop, M., & Colman, R. F. (1973) *J. Biol. Chem. 248*, 7359.

Smith, E. L., Landon, M., Piszkiewicz, D., Brattin, W. J., Langley, J. J., & Melamed, M. D. (1970) *Proc. Natl. Acad. Sci. U.S.A. 67*, 724.