Affinity Labeling of a Regulatory Site of Bovine Liver Glutamate Dehydrogenase†

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ABSTRACT: A new adenosine analog, 3′-*p*-fluorosulfonylbenzoyladenosine (3′-FSBA), has been synthesized which reacts covalently with bovine liver glutamate dehydrogenase. Native glutamate dehydrogenase is activated by ADP and inhibited by high concentrations of DPNH. Both of these effects are irreversibly decreased upon incubation of the enzyme with the adenosine analog, 3′-*p*-fluorosulfonylbenzoyladenosine (3′-FSBA), while the intrinsic enzymatic activity as measured in the absence of regulatory compounds remains unaltered. A plot of the rate constant for modification as a function of the 3′-FSBA concentration is not linear, suggesting that the adenosine derivative binds to the enzyme ($K_I = 1.0 \times 10^{-4}$ M) prior to the irreversible modification. Protection against modification by 3′-FSBA is provided by ADP and by high concentrations of DPNH, but not by the inhibitor GTP, the substrate α-keto glutarate, the coenzyme TPNH, or low concentrations of the coenzyme DPNH. The isolated altered enzyme contains approximately 1 mol of sulfonylbenzoyladenosine per peptide chain, indicating that a single specific regulatory site has reacted with 3′-FSBA. The modified enzyme exhibits normal Michaelis constants for its substrates and is still inhibited by GTP, albeit at a higher concentration, but it is not inhibited by high concentrations of DPNH. Although ADP does not appreciably activate the modified enzyme, it does (as in the case of the native enzyme) overcome the inhibition of the modified enzyme by GTP. These results suggest that ADP can bind to the modified enzyme, but that its ability to activate is affected indirectly by the modification of the adjacent DPNH inhibitory site. It is proposed that the regulatory sites for ADP and DPNH are partially overlapping and that 3′-FSBA functions as a specific affinity label for the DPNH inhibitory site of glutamate dehydrogenase. It is anticipated that 3′-*p*-fluorosulfonylbenzoyladenosine may act as an affinity label of other dehydrogenases as well as of other classes of enzymes which use adenine nucleotides as substrates or regulators.

Bovine liver glutamate dehydrogenase (L-glutamate: $NAD(P)^+$ oxidoreductase (deaminating), ED 1.4.1.3) is a major regulatory enzyme whose activity is modulated by purine nucleotides. Kinetic as well as direct binding studies have indicated that certain activating nucleotides, such as ADP, and certain inhibitory nucleotides, such as GTP, bind to each polypeptide chain in a manner that is mutually exclusive, although they do not occupy identical sites (Goldin and Frieden, 1972; Fisher, 1973). The enzyme is also inhibited by relatively high concentrations of DPNH which bind to a second nonactive site (Frieden, 1959). There has been some controversy about the relationship between this inhibitory DPNH and the activating ADP sites: it was initially proposed that the second DPNH site was distinct from the "purine nucleotide" site occupied by ADP and GTP (Frieden, 1963); however, more recent experiments have implied that ADP, but not GTP, may compete for binding to the adenyl portion of the nonactive site of DPNH (Pantaloni and Dessen, 1969; Cross and Fisher, 1970; Koberstein *et al.*, 1973; Pantaloni and Lecuyer, 1973).

A number of attempts have been made to identify, by means of chemical modification, particular amino acid residues which participate in the regulatory sites of glutamate dehydrogenase. For example, treatment of tyrosyl residues of the enzyme with *N*-acetylimidazole or tetranitromethane decreases its response to inhibition by GTP (Price and Radda, 1969; Smith *et al.*, 1970; Smith and Piszkiewicz, 1973); modification of lysyl and tyrosyl residues with fluorodinitrobenzene produces desensitization toward ADP and GTP as well as loss of catalytic activity (diPrisco, 1971); and reaction of lysyl residues with trinitrobenzenesulfonate (Goldin and Frieden, 1971; Clark and Yielding, 1971), with acetic anhydride (Colman and Frieden, 1966a,b) and with pyridoxal phosphate (Smith *et al.*, 1970; Goldin and Frieden, 1972), leads to loss of inhibition by DPNH, altered affinity for ADP and GTP and inactivation. The catalytic and various regulatory regions must be closely interrelated in the structure of this enzyme, and the interpretation of chemical modification studies conducted with reactive and relatively nonspecific reagents has been complicated in most cases by multiple sites of reaction which accordingly produce multiple effects on the properties of the enzyme. Affinity labeling, whereby a chemical modifying reagent is bound at a regulatory site on the enzyme prior to covalent reaction, is an approach which may lead to more specific modification of the enzyme which in turn may yield results more amenable to interpretation in terms of the amino acid residues involved in particular sites. This paper reports the preparation of a new adenosine analog containing an alkylating functional group: 3′-fluorosulfonylbenzoyladenosine (3′-FSBA).[1] The stoichiometric reaction of this compound at the inhibitory DPNH site of glutamate dehydrogenase is described, as well as the effects of this modification on the

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[1] Abbreviations used are: 3′-FSBA, 3′-*p*-fluorosulfonylbenzoyladenosine; 3′-SBA, 3′-sulfonylbenzoyladenosine.

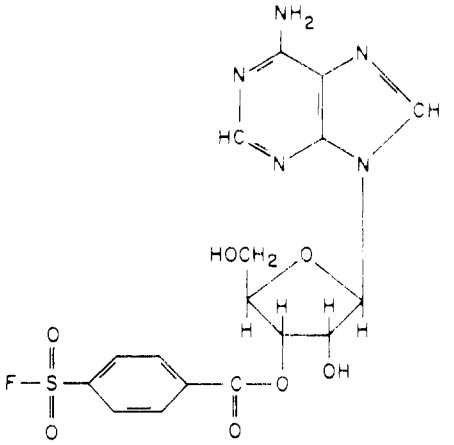

FIGURE 1: Structure of 3′-*p*-fluorosulfonylbenzoyladenosine.

kinetic characteristics of the enzyme. A preliminary version of this work has been presented (Pal *et al.*, 1974).

Experimental Procedure

Materials. Bovine liver glutamate dehydrogenase was purchased from Boehringer-Mannheim as a crystalline suspension in ammonium sulfate. About 40 mg was dialyzed for 16 hr at 4° against two changes of 0.1 M potassium phosphate buffer (pH 7.12). The dialyzed enzyme was then centrifuged at 4° for 30 min at 20,000 rpm in a Sorvall Model RC2-B centrifuge and the enzyme solution was carefully separated from the precipitated denatured protein. The A_{280}:A_{260} ratio was about 1.9 and the enzyme concentration was determined using the value $E^{1\%} = 9.7$ (Olson and Anfinsen, 1952). The enzyme was stored in frozen aliquots at −85° and was thawed immediately before each experiment. A molecular weight of 56,100 for the identical polypeptide chains of glutamate dehydrogenase was used for all calculations (Smith *et al.*, 1970).

The coenzymes DPNH and TPNH and nucleotides ADP and GTP were obtained from Sigma Chemicals. Both DPNH and TPNH solutions were prepared the same day as used.

Preparation of 3′-p-Fluorosulfonylbenzoyladenosine (3′-FSBA). The reagent 3′-fluorosulfonylbenzoyladenosine (3′-FSBA), shown in Figure 1, was prepared by reaction of adenosine with fluorosulfonylbenzoyl chloride. Adenosine (4.02 g, 0.015 mol) was dissolved in 75 ml of dry dimethylformamide and 1.86 g (0.015 mol) of the base 1,5-diazabicyclo[3.4.0]nonene-5 was added to accept the mole of HCl from the reaction of the acid chloride with the alcohol. This base was employed because it is a particularly good HCl scavenger. Other bases, including triethylamine and pyridine, were also used successfully. The mixture was stirred at room temperature for 2 hr and then 3.33 g (0.015 mol) of *p*-fluorosulfonylbenzoyl chloride was added and the solution was allowed to stand for 44 hr. The solvent was evaporated *in vacuo* to yield a glassy residue. This material was redissolved in a few milliliters of dimethylformamide and the crude product was precipitated as an oil by the addition of ethyl acetate–ether (1:1). The product was triturated with this solvent and then converted to a solid by trituration with water. The solid was collected, washed with water, and dried; weight, 4.3 g. Thin-layer chromatography on silica gel, using as solvent methyl ethyl ketone–acetone–water (65:20:15) showed a small amount of a product of R_F 0.93, and substantial quantities of products with R_F's of 0.78 and 0.86.

The products were separated by chromatography over silica gel (Merck-Darmstadt, 0.05–0.2 mm). The column size was 4.6 cm × 100 cm and the solvent used was methyl ethyl ketone–acetone–H_2O, 72:20:8. The crude product was dissolved in 100 ml of solvent and applied to the column with several washes. The effluent was monitored by spotting aliquots on fluorescent thin-layer plates and examining them for uv absorbing material. After 850 ml of effluent, uv absorbing material began to emerge. At this point, 20-ml fractions were collected. The separation was monitored by thin-layer chromatography. The two main products were recovered from fractions 18–23 (R_F 0.81, wt, 0.86 g) and 28–34 (R_F 0.70, wt, 1.02 g). The material of lower R_F was recrystallized from ethanol–ethyl acetate to obtain 0.39 g, mp 201–202°.

By a combination of elemental analysis, nuclear magnetic resonance (nmr), and ir spectra, it was established that the material of lower R_F was 3′-fluorosulfonylbenzoyladenosine. A sample was dried *in vacuo* at 25° for analysis. *Anal.* Calcd for $C_{17}H_{16}N_5O_7FS \cdot 2H_2O$: C, 41.72; H, 4.12; N, 14.31; F, 3.88; S, 6.55. Found: C, 41.83; H, 3.40; N, 14.03; F, 4.06; S, 6.92. The nmr spectrum measured in dimethylformamide gave resonance peaks which were assigned to the adenosine protons as follows: 5′ α + β-AB of ABX centered at δ 3.98; 4′ apparent d of d δ 4.61; 2′ d of d δ 5.27; 3′ d of d δ 5.83, $J_{3,4}$ = 1.9 Hz; 1′ d δ 6.32, $J_{1,2}$ = 7.2 Hz; NH_2 broad S δ 7.50; 2 OH's broad S δ 6.3; 2 or 8 S δ 8.30; and 2 or 8 S δ 8.48. That the product is one material follows from the integrated areas of all protons in the 100-MHz spectrum. Doubtless a small amount of 2′ ester (less than 5%) could not have been detected. One methine proton is shifted from the envelope usually containing both 2′ and 3′ protons. That this shifted proton is not H(2′) follows from the $J_{3,4}$ coupling of 1.9 Hz, which is clearly different from the $J_{1,2}$ coupling of 7.2 Hz. Therefore, the resonance at 5.83 must be H(3′). The identification of this compound as 3′-fluorosulfonylbenzoyladenosine rests on the resonances of the critical protons and the high value for the $J_{1,2}$ coupling. The identification of this compound was made in comparison with 2′,3′,5′-tris-*p*-fluorosulfonylbenzoyladenosine, 2′,3′-bis-*p*-fluorosulfonylbenzoyladenosine, and 5′-fluorosulfonylbenzoyladenosine. The infrared spectrum was consistent with the identification. The ultraviolet absorption spectrum exhibits maxima at 232 nm (ϵ 2.01 × 10^4) and 258 nm (ϵ 1.63 × 10^4) when measured in ethanol.

The elemental analysis, as well as nmr and infrared spectra, revealed that the material of higher R_F was 2′,3′-bis-*p*-fluorosulfonylbenzoyladenosine.

Enzymatic Assay. Enzymatic activity was determined at 25° in Tris–0.01 M acetate buffer (pH 8) containing 10 μM EDTA, by measuring the oxidation of reduced coenzyme at 340 nm using a Gilford Model 240 spectrophotometer equipped with an expanded scale recorder (0–0.1 absorbance full scale). 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 activity was measured in the presence of ADP, the concentration of the nucleotide was 100 μM unless indicated otherwise. The amount of DPNH or TPNH oxidized was calculated by using the extinction coefficient at 340 nm (ϵ 6.22 × 10^3 l. mol^{-1} cm^{-1}). The Michaelis constants were also determined at pH 8 with two substrates being held constant at the concentrations given above and the third being varied.

Reaction of Glutamate Dehydrogenase with 3′-FSBA. Glutamate dehydrogenase was incubated with 3′-*p*-fluoro-

sulfonylbenzoyladenosine at 24° in 0.01 M sodium barbital buffer (pH 8) containing 0.43 M KCl. During the course of the reaction, aliquots were withdrawn at a given time, diluted 20-fold with Tris–0.1 M acetate buffer (pH 8) at 0°, and assayed in the presence and absence of ADP.

In addition to reacting with enzyme, the compound 3′-FSBA was also observed to react with buffer. As a result of this reaction 3′-FSBA loses its ability to modify the enzyme, and undergoes a spectral change, exhibiting an increase in absorbance at 248 nm and a decrease in absorbance at 293 nm, while the absorbance at 258 nm remains essentially constant. The spectral change is accompanied by a release of fluoride ion, as determined with a fluoride specific electrode (Beckman). The buffer reaction as monitored by either the spectral change or the release of fluoride ion follows first-order kinetics. In the case of 0.01 M potassium phosphate and 0.01 M Tris-acetate buffers at pH 8, the half-life is about 37 min and for 0.01 M sodium barbital buffer (pH 8) the half-life is around 63 min. Triethanolamine chloride buffer (pH 8) was found to react with the reagent more vigorously. Therefore, 0.01 M sodium barbital buffer (pH 8) was selected for the study of the reaction of 3′-FSBA with glutamate dehydrogenase.

Preparation of the Modified Enzymes. Glutamate dehydrogenase (2 mg/ml) was incubated with 3′-*p*-fluorosulfonylbenzoyladenosine (0.482 mM) in 0.01 M sodium barbital buffer (pH 8) containing 0.43 M KCl for 2 hr, after which the reaction mixture was dialyzed for 16 hr at 4° against two changes of 0.1 M potassium phosphate buffer (pH 7.12). The dialyzed enzyme solution was centrifuged for 30 min at 20,000 rpm to remove any denatured protein. The concentration of the modified enzyme was determined by the Biuret method. The modified enzyme was kept frozen in aliquots. In the preparation of the protected modified enzymes, the reaction mixture consisted of glutamate dehydrogenase (2 mg/ml), 3′-FSBA (0.482 mM), and either ADP (0.85 mM) or DPNH (3 mM) in 0.01 sodium barbital buffer (pH 8) containing 0.43 M KCl. After 2 hr of incubation at 24°, these modified enzymes were dialyzed and centrifuged as in the previous case.

Moles of 3′-FSBA Incorporated Per Subunit. Three approaches were used to determine the number of moles of sulfonylbenzoyladenosine incorporated per peptide chain of the enzyme. The first, a spectroscopic method; the second, a centrifugation technique; and the third, an electrochemical method. In the spectroscopic method, the number of moles of 3′-FSBA bound per subunit polypeptide chain was determined from the difference in absorbance at 258 nm between the isolated 3′-FSBA-modified enzyme and native glutamate dehydrogenase. The extinction coefficient of free 3′-FSBA at 258 nm (1.63×10^4 l. mol^{-1} cm^{-1}) was used in this calculation, with the implicit assumption that this extinction coefficient is not appreciably affected by the covalent reaction of 3′-FSBA with the enzyme. The concentrations of the native and modified enzymes were determined by the Biuret method.

In the centrifugal method, 1 ml of mixture containing glutamate dehydrogenase (2 mg/ml = 35.6 μM), 3′-FSBA (0.240 mM), and KCl (0.43 M) in 0.01 M sodium barbital buffer (pH 8) was incubated at 24° for 2 hr, after which the protein was precipitated by the addition of 1 ml of saturated Na_2SO_4. The free 3′-FSBA was isolated from enzyme-bound 3′-FSBA by centrifugation at 25,000 rpm for 30 min. The supernatant was carefully separated and the precipitate was redissolved in 0.5 ml of sodium barbital buffer containing 0.43 M KCl, allowed to stand for 15 min, and then reprecipitated with 0.5 ml of saturated Na_2SO_4. This process was repeated three times and the supernatants were pooled together. From the total volume of the supernatant and the known value of the extinction coefficient of 3′-FSBA at 277 nm, the amount of free 3′-FSBA could be determined. A control experiment was performed using identical reaction mixtures except for the absence of 3′-FSBA. The supernatant of the control sample was checked for the presence of any unprecipitated protein (which was less than 1%) and the absorbance at 277 nm of the experimental sample was corrected for protein absorption. The number of moles of enzyme-bound 3′-FSBA was calculated from the difference between the total reagent in the original reaction mixture and the measured amount of free 3′-FSBA, with the moles of peptide chain being calculated from the amount of glutamate dehydrogenase initially present in the reaction mixture.

In the electrochemical method, the extent of reaction was assessed by the release of fluoride ion from 3′-FSBA. Glutamate dehydrogenase (2 mg/ml) was incubated with 3′-FSBA (0.482 mM) in 0.01 M sodium barbital buffer (pH 8) containing 0.43 M KCl, as above. The moles of fluoride released was determined by means of a specific fluoride electrode (Beckman) after 75 min when the reaction was approximately 89% complete. Corrections were made for the decomposition of the reagent in the sodium barbital buffer by measuring the moles of fluoride ion released upon incubation of 3′-FSBA in the identical solution except for the absence of enzyme. A standard curve for the fluoride electrode was initially established by measuring the potential of solutions of sodium fluoride over the concentration range 0.01–10 mM, dissolved in 0.01 M sodium barbital buffer (pH 8) containing 0.43 M KCl.

Results

Reaction of Glutamate Dehydrogenase with 3′-Fluorosulfonylbenzoyladenosine. The maximum velocity of native glutamate dehydrogenase is increased about 2.5-fold by added ADP when assayed in the presence of TPNH as coenzyme (Figure 2). As a result of incubation of glutamate dehydrogenase with 3′-fluorosulfonylbenzoyladenosine, the ability of the enzyme to be activated by ADP is progressively lost, although the intrinsic activity of the enzyme in the absence of any modifying compounds remains constant (Figure 2). After 80 min of incubation, the extent of activation by added ADP is reduced to 1.33 times the rate in the absence of added nucleotides. Prolongation of the incubation time or increases in the reagent concentration do not significantly alter the magnitude of activation by added ADP, suggesting that ADP can still bind to the modified enzyme but that it is less effective in enhancing the catalytic activity of that enzyme. The time-dependent decrease in the ADP activation obeys pseudo-first-order kinetics, as shown in the insert of Figure 2, yielding a rate constant of 0.0351 min^{-1} at a 3′-FSBA concentration of 0.496 mM.

The pseudo-first-order rate constant for modification of glutamate dehydrogenase was measured as a function of 3′-FSBA concentration over the range 0.0992–0.496 mM. Concentrations less than 0.0992 mM were not used because below this level the reaction rate of enzyme with 3′-FSBA becomes comparable to the reaction rate of buffer with the reagent. A plot of the observed rate constant *vs.* 3′-FSBA concentration is not linear, but rather exhibits saturation kinetics. The simplest explanation of this type of kinetic be-

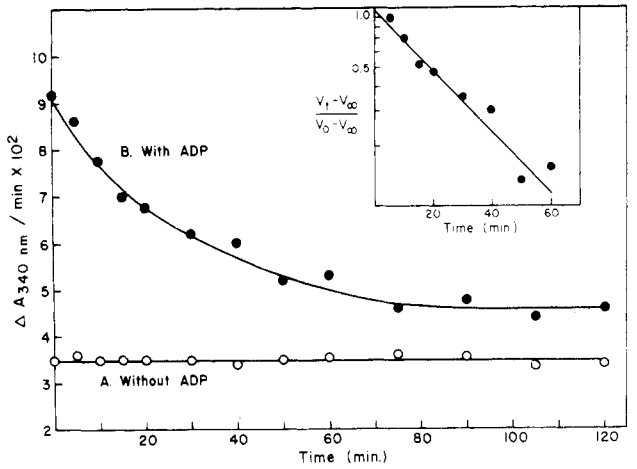

FIGURE 2: Reaction of 3′-*p*-fluorosulfonylbenzoyladenosine with glutamate dehydrogenase. Glutamate dehydrogenase (0.21 mg/ml) was incubated with 3′-FSBA (0.496 mM) at 24° in 0.01 M sodium barbital buffer (pH 8) containing 0.43 M KCl. At each indicated time, a 30-μl aliquot was withdrawn, diluted 20-fold with Tris–0.1 M acetate buffer (pH 8) at 0°, and assayed (A) in the absence and (B) in presence of 100 μM ADP as described in Experimental Procedure, using 100 μM TPNH as coenzyme. Insert: Determination of the pseudo-first-order rate constant from the decrease in activation caused by ADP. (V_t and V_0 are the enzymatic velocities measured in the presence of ADP at the given and zero time, respectively, and V_∞ is the constant velocity at the end of the reaction. The pseudo-first-order rate constant calculated is 0.0351 min^{-1}.)

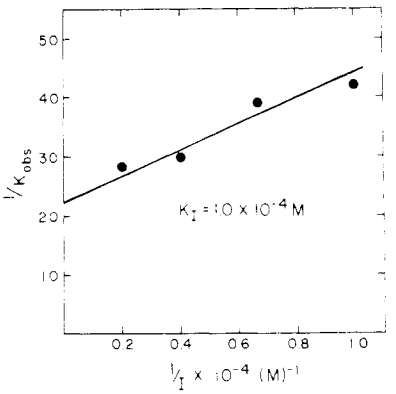

FIGURE 3: Effect of concentration of 3′-*p*-fluorosulfonylbenzoyladenosine on the pseudo-first-order rate constant of reaction. Glutamate dehydrogenase (0.21 mg/ml) was incubated with different concentrations of 3′-FSBA at 24° in 0.01 M sodium barbital buffer (pH 8) containing 0.43 M KCl.

havior is that the reagent (I) binds reversibly to the enzyme to form an enzyme–3′-FSBA complex (EI), prior to the irreversible covalent modification reaction to yield altered enzyme (E′). The observed rate constant for modification can be expressed as

$$E + I \overset{K_I}{\rightleftharpoons} EI \xrightarrow{k} E'$$

$$k_{obsd} = \frac{k}{1 + [K_I/(I)]} \quad (1)$$

where k_{obsd} is the apparent rate constant observed at a particular concentration of 3′-FSBA, K_I is the dissociation constant of the enzyme–3′-FSBA complex, and k is the intrinsic rate constant for covalent modification of the enzyme observed at infinitely high concentrations of the reagent. The reciprocal form of eq 1 is

$$\frac{1}{k_{obsd}} = \frac{1}{k} + \frac{K_I}{k}\left(\frac{1}{[I]}\right). \quad (2)$$

Table I: Rate of Reaction of 3′-*p*-Fluorosulfonylbenzoyladenosine with Glutamate Dehydrogenase in the Presence of Substrates and Modifiers.[a]

Additions to Reaction Mixture	k ($min^{-1} \times 10^3$)
None	27.5
α-Ketoglutarate (20 mM)	27.2
GTP (0.0915 mM)	27.2
ADP (0.25 mM)	4.8
ADP (0.80 mM)	No reaction
TPNH (1.0 mM)	37.6
DPNH (0.1 mM)	32.2
DPNH (1.06 mM)	8.1
DPNH (3.18 mM)	No reaction

[a] Glutamate dehydrogenase (0.21 mg/ml) was incubated with 3′-FSBA (0.145 mM) at 24° in 0.01 M sodium barbital buffer (pH 8) and the ionic strength was adjusted to 0.43 M by the addition of KCl. The pseudo-first-order rate constants for the reaction were determined according to Figure 2 and Experimental Procedure.

A plot of $1/k_{obsd}$ *vs.* $1/(I)$ yields a straight line, as shown in Figure 3, which allows the calculation of K_I as 0.100 mM. This indication of the formation of the EI complex implies the existence of a specific interaction between the enzyme and 3′-FSBA which may be responsible for the special ability of the adenosine analog to react with glutamate dehydrogenase. That this interaction requires the adenosine portion of the molecule is shown by the fact that whereas 3′-FSBA exhibits a half-life for modification of 25 min at 0.145 mM, the compound *p*-fluorosulfonylbenzoic acid, when incubated with the enzyme at the same concentration and under the same conditions, fails to alter the kinetic behavior of the enzyme after 2 hr.

Table I records the effect on the rate constant for modification by 0.145 mM 3′-FSBA of the addition of substrates and modifiers to the reaction mixture. The substrate, α-ketoglutarate, when present at a concentration ten times the value of its Michaelis constant, does not influence the rate constant. This result suggests that the locus of reaction of 3′-FSBA does not lie within the substrate binding site of the enzyme. Similarly, the rate constant is not decreased by the allosteric inhibitor GTP although the nucleotide is present at concentrations in considerable excess of its dissociation constant. In contrast, the presence of the allosteric activator ADP produces striking protection against the kinetic effects of modification, implying that 3′-FSBA reacts with the enzyme at or near the binding site for ADP. DPNH is known to bind not only to the catalytic coenzyme site of glutamate dehydrogenase (as does TPNH), but also to a second inhibitory site (Frieden, 1959). This allosteric inhibitory site specifically requires DPNH, and does not bind TPNH appreciably. Table I shows that relatively high concentrations of DPNH, but not TPNH, protect against the alteration of the kinetic characteristics of the enzyme which are produced by 3′-FSBA. The distinction between the effects of 1 mM DPNH and TPNH argues against the possibility that the protection provided by DPNH or ADP are artifacts produced by direct reaction of the amino group of the adenine moiety with 3′-FSBA. Rather, it appears that the site of attack of the enzyme by 3′-FSBA is in the vicinity of the second DPNH inhibitory site as well as of the allosteric ADP site.

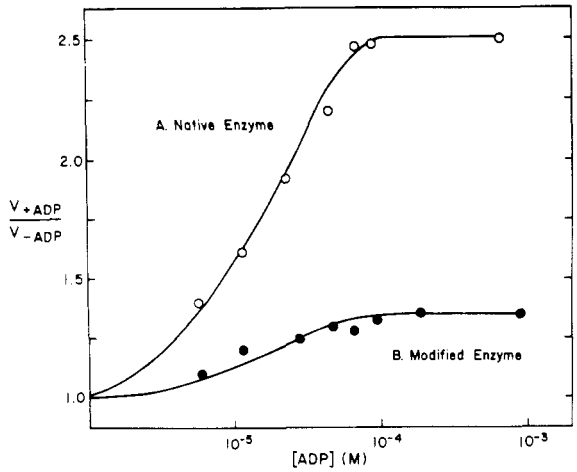

FIGURE 4: The ratio of velocities in the presence and absence of ADP as a function of ADP concentration of (A) native and (B) modified enzyme. The velocities were measured under the conditions described in Experimental Procedure, using 100 μM TPNH as coenzyme. ADP was added as indicated.

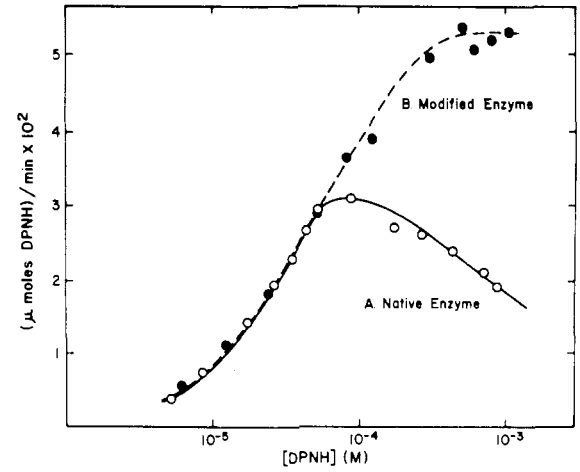

FIGURE 5: Velocity as a function of DPNH concentration of (A) native and (B) modified enzyme. At DPNH concentrations up to 2 × 10^{-4} M velocity measurements were made at 340 nm, as described in Experimental Procedure. At DPNH concentrations higher than 2 × 10^{-4} M, measurements were made at 375 nm and the amount of coenzyme oxidized was calculated using the value of ϵ^{375} = 1.85 × 10^3 l. mol^{-1} cm^{-1} for DPNH.

Stoichiometry of Incorporation by 3′-FSBA. In view of the observed protection by two distinct allosteric modifiers, it was important to establish whether the 3′-FSBA was reacting with one or more amino acid residues of the enzyme. As detailed in Experimental Procedure, the stoichiometry of incorporation of 3′-FSBA was determined by three methods. The first approach was based on the spectral difference between modified and native glutamate dehydrogenase. The modified enzyme exhibits enhanced absorbance at wavelengths below 280 nm. Using the difference in absorbance between the native and modified enzymes at 258 nm and the extinction coefficient for *free* 3′-FSBA, it can be calculated that 1.31 mol of 3′-sulfonylbenzoyladenosine are incorporated per peptide chain of glutamate dehydrogenase when the enzyme has reacted completely.

Alternatively, the moles of bound 3′-sulfonylbenzoyladenosine can be calculated from the difference between the total moles of 3′-FSBA incubated with the enzyme and the residual unreacted 3′-FSBA measured after precipitation of the fully modified glutamate dehydrogenase. This technique yields a value of 0.86 mol of 3′-sulfonylbenzoyladenosine incorporated per peptide chain.

The stoichiometry can also be determined from the moles of fluoride ion released from 3′-FSBA during the course of reaction with a known amount of enzyme. In this case it was observed that 0.85 mol of fluoride was released per peptide chain concomitant with modification of the enzyme to the extent of 89%. On the basis of the three techniques used, it may be concluded that the incorporation of approximately 1 mol of 3′-sulfonylbenzoyladenosine is responsible for the alteration of the properties of glutamate dehydrogenase.

Catalytic Characteristics of Modified Enzyme. The kinetic properties of modified enzyme, containing approximately 1 mol of covalently bound 3′-sulfonylbenzoyladenosine per peptide chain, were examined in detail in comparison with those of native glutamate dehydrogenase. It was noted earlier that reaction of 3′-FSBA with the enzyme was accompanied by a marked reduction in the extent of activation by 100 mM ADP. Indeed, this altered characteristic was used to monitor the reaction of enzyme with 3′-FSBA. However, the decreased activation at a constant concentration can be attributed to either a weakened affinity of the enzyme for ADP or to a decreased maximum extent of activation. In order to distinguish between these possibilities, the concentration dependence of activation by ADP was tested under the same conditions for native and modified enzyme. In accordance with the relationship for an uncompetitive allosteric modifier described by Frieden (1963), the dissociation constant for the enzyme–ADP complex (K_{ADP}) was numerically equal to the concentration of ADP at which the maximum velocity is given by

$$(V_0 + V_A)/2 \qquad (3)$$

where V_0 = the maximum velocity observed under the given set of conditions in the absence of ADP, and V_A = the maximum velocity observed under the same conditions but in the presence of a saturating concentration of ADP. The curves, shown in Figure 4, yield dissociation constants of 15 and 17 μM for the native and modified enzymes, respectively; whereas, the maximum extent of activation by ADP (*i.e.*, V_A/V_0) is 2.5 for the native enzyme, but only 1.3 for the modified enzyme. Thus, modification produces a marked decrease in the degree of activation, without influencing the affinity for ADP.

Figure 5 compares for the native and modified enzymes the dependence of velocity on the initial concentration of DPNH in the assay solution. The two curves coincide at concentrations up to 5 × 10^{-5} M. Indeed, a Lineweaver–Burk plot of the data for curves A and B yields the same extrapolated maximum velocity. A Michaelis constant of 5.62 × 10^{-5} M is obtained from the linear plot for the modified enzyme, and the comparable Michaelis constant (K_{DPNH-1}) of 6.66 × 10^{-5} M is calculated from the linear portion of the double reciprocal plot for the native enzyme. Above a concentration of 5 × 10^{-5} M DPNH inhibition is observed for the native enzyme only, as has been reported previously (Frieden, 1959). The DPNH inhibition constant (K_{DPNH-2}), calculated from the relationship (Frieden, 1959)

$$v_{obsd} = \frac{V_{max}}{1 + \frac{K_{DPNH-1}}{(DPNH)} + \frac{(DPNH)}{K_{DPNH-2}}} \qquad (4)$$

is approximately 3.36 × 10^{-4} M for the native enzyme. In

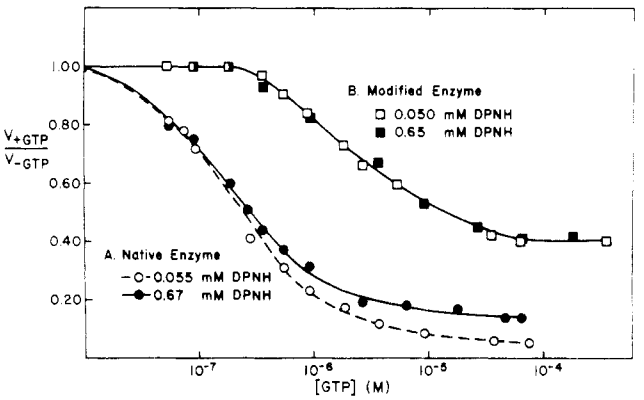

FIGURE 6: Plot of the ratio of velocities in presence and in absence of GTP. The velocities were measured at two DPNH concentrations, for (A) native and (B) modified enzyme. At the lower DPNH concentration, absorbance measurements were made at 340 nm, while at the higher DPNH concentration measurements were made at 375 nm.

striking contrast, no inhibition is observed for the modified enzyme at high concentrations of DPNH; rather, this enzyme exhibits normal Michaelis-Menten kinetics over the entire range of coenzyme concentration. The difference between the two curves of Figure 5 has been confirmed by conducting the incubation of enzyme with 3′-FSBA under the conditions described in Figure 2, but by measuring the velocity of aliquots of the incubation mixture at various time intervals in the presence of 3×10^{-4} M DPNH. At this concentration the native enzyme exhibits considerable inhibition, whereas the modified enzyme is operating at almost its maximum velocity. During the 2-hr period of incubation of enzyme with the reagent, the velocity observed in the presence of 3×10^{-4} M DPNH increases twofold, which coincides with the difference between the curves for native and fully modified enzyme shown in Figure 5. It would be equally as possible to monitor the rate of reaction of 3′-FSBA with the enzyme under various conditions by the loss of inhibition by DPNH, as by the decrease in activation by ADP. Reaction of glutamate dehydrogenase with 3′-FSBA thus appears to eliminate the inhibition produced by binding of DPNH to a second site.

Glutamate dehydrogenase is known to be profoundly inhibited by GTP (Goldin and Frieden, 1972a). It was initially reported that at high concentrations of DPNH the dissociation constant for the enzyme-GTP complex was smaller than at low concentrations of DPNH (Frieden, 1963). However, as shown in Figure 6A, the sensitivity of native enzme to GTP inhibition is not significantly different at 0.055 mM as compared to 0.67 mM DPNH. This observation is in agreement with the observation that native glutamate dehydrogenase exhibits essentially the same binding constant for radioactive GTP over the DPNH concentration range of 0.10–0.80 mM (Colman and Frieden, 1966c). From the data of Figure 6, the dissociation constants for the enzyme-GTP complex (K_{GTP}), in analogy to K_{ADP}, were numerically equal to the GTP concentration at which the maximum velocity is given by

$$(V_0 + V_I)/2 \qquad (5)$$

where V_0 = the maximum velocity observed in the absence of GTP, and V_I = the maximum velocity observed under the same conditions but in the presence of a saturating concentration of GTP. Values of 0.22 and 0.24 μM were obtained for the native enzyme at 0.055 and 0.67 mM DPNH, respectively. The maximum extent of inhibition is 5–7%. In

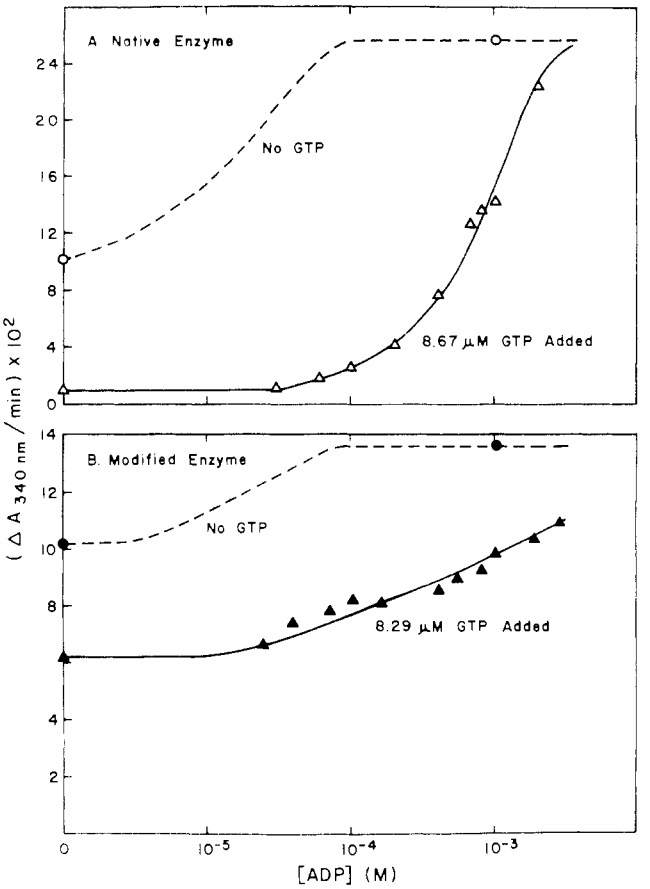

FIGURE 7: Plot of velocity in the presence of 8 μM GTP as a function of ADP concentration. Curve A records data for native and curve B for modified enzyme. To facilitate comparison, the data for velocity as a function of ADP concentration in the absence of GTP were taken from Figure 4, normalized with respect to enzyme concentration, and plotted as the dotted curves.

contrast, the modified enzyme exhibits both a weakened affinity for the inhibitor (K_{GTP} = 2.4 μM) and a decreased maximum extent of inhibition (40%). This weakened affinity for GTP was also observed when the coenzyme in the assay was TPNH.

The purine nucleotide modifiers of glutamate dehydrogenase, GTP and ADP, exhibit kinetic competition. This observation is illustrated for native enzyme in Figure 7A, in which the inhibition produced by 8 μM GTP is overcome by increasing concentrations of ADP in the assay solution; conversely, a higher concentration of ADP is required in order to activate the enzyme when GTP is present as compared to the case when it is absent. Figure 7B demonstrates that although ADP does not appreciably activate the modified enzyme, it is still able to overcome the inhibition produced by GTP. This result constitutes strong evidence that the modified enzyme can bind ADP, but that its ability to be activated by this nucleotide is disrupted by reaction of the enzyme with 3′-fluorosulfonylbenzoyladenosine.

The affinity of glutamate dehydrogenase for the substrates α-ketoglutarate and NH_4^+ is not altered by modification by 3′-FSBA. Thus, K_m values of 1.3 and 2.0 mM for α-ketoglutarate and NH_4^+, respectively, were measured for the modified enzyme in the presence of TPNH, whereas the corresponding values for the native enzyme are 1.9 and 2.6 mM (Colman and Frieden, 1966a). Similarly, the Michaelis constants for the catalytically significant DPNH site have comparable values (56 μM for modified and 67 μM for native enzyme).

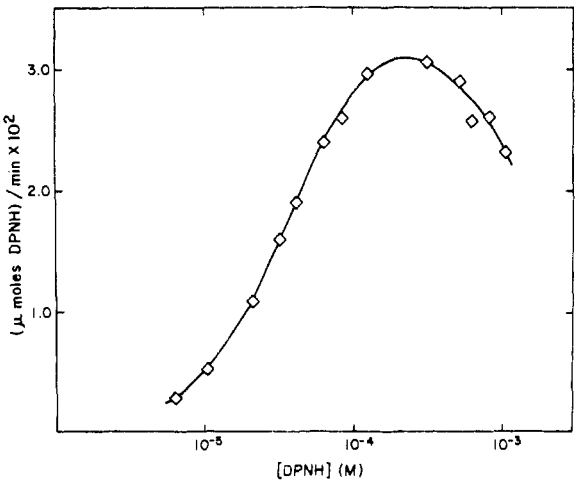

FIGURE 8: Plot of velocity as a function of DPNH concentration of ADP-protected modified enzyme. When DPNH concentrations were higher than 2×10^{-4} M, measurements were done at 375 nm.

Enzymes Treated with 3′-FSBA in Presence of DPNH or ADP. As indicated in Table I, glutamate dehydrogenase is protected against the 3′-FSBA produced loss of sensitivity to activation by ADP by including either ADP or high concentrations of DPNH in the reaction mixture. Upon isolation of these "protected" enzymes, as described in Experimental Procedure, it was found by the spectroscopic method that glutamate dehydrogenase protected with ADP contained 1.23 groups of 3′-SBA per peptide chain, while the enzyme protected with DPNH contained 1.11 groups of 3′-SBA per peptide chain. Figure 8 demonstrates that the ADP-protected enzyme exhibits inhibition by high concentrations of DPNH. A curve that was almost superimposable with that of Figure 8 was obtained for the DPNH-protected enzyme. Since these protected proteins are also activatable by ADP, the 3′-FSBA cannot in these enzymes react at precisely the same site as in modified enzyme prepared without added ADP or DPNH.

Discussion

In affinity labeling, the specificity of the enzyme for a substrate analog is exploited in order to limit the chemical modification to the region of the active site. This approach is well suited for the specific chemical modification of regulatory sites, in which the participating amino acid residues are not necessarily expected to be particularly reactive. The compound 3′-fluorosulfonylbenzoyladenosine exhibits features which are favorable for the exploration of a purine nucleoside binding site. It has an intact adenine ring, and therefore should bind effectively and specifically to adenine (as contrasted to guanine) binding sites on proteins. The introduction into the purine ring of bulky reactive functional groups may either interfere with the binding of the compound or necessitate its binding at a different location on the enzyme from the usual adenine site. This type of explanation may account for the absence of any observed changes in the kinetic properties of glutamate dehydrogenase after incubation with 6-(2′,3′-*O*-isopropylidenepurine ribonucleoside)-5-(2-nitrobenzoic acid) thioether or *S*-(2,4-dinitrophenyl)-6-mercaptopurine ribonucleoside 5′-monophosphate (Worthen and Colman, unpublished observations), which were reported by Hulla and Fasold (1972), to react covalently with and activate phosphorylase *b*. Similarly, defective or abnormal binding might be expected for some enzymes in the case of interaction with N^6-(ethyl 2-diazomalonyl) cAMP which was prepared as a photoaffinity label by Brunswick and Cooperman (1971). In addition to an intact adenine ring, 3′-FSBA possesses a ribose moiety and thus is closer in structure to the normal ligand of the enzyme than are certain of the purine derivatives which were previously designed as affinity labels (Schaeffer and Oden, 1966; Anderson and Graves, 1973). The 3′ position of ribose is not generally derivatized in purine nucleosides which bind to the substrate and regulatory sites of enzymes and thus for those enzymes which can tolerate the added bulk in the vicinity of the 3′ position, 3′-FSBA may function in accordance with Baker's (1967) definition of an exoalkylating agent: one which participates in covalent bond formation with an enzymic nucleophilic group located in a position immediately adjacent to, if not actually within, the substrate or regulator binding site. In contrast to the specificity of the adenosyl portion of 3′-FSBA, the sulfonyl halide can act as an electrophilic agent capable of reacting covalently with a number of classes of amino acids, particularly serine, tyrosine, lysine, and histidine (Poulos and Price, 1974). Once bound to an adenosyl site on an enzyme, there is thus a reasonable probability that 3′-FSBA would be able to react with an amino acid which has a nucleophilic functional group in the region to which the ribose moiety of 3′-FSBA is bound. It is anticipated that 3′-FSBA will function as an affinity label for a variety of enzymes which have adenosyl binding sites as part of their catalytic or regulatory sites; these enzymes might include dehydrogenases as well as kinases.

Glutamate dehydrogenase provides an excellent test system for assessing the usefulness of 3′-FSBA as an affinity label for purine nucleotide binding sites since this enzyme has a catalytic as well as a nonactive inhibitory site for DPNH, and at least partially discrete regulatory sites for ADP and GTP (Goldin and Frieden, 1972a). It has also been shown that adenosine, as well as its phosphorylated derivatives, are capable of binding to the enzyme (Cross and Fisher, 1970). The results presented in this paper indicate that 3′-FSBA meets the criteria for an effective affinity label for glutamate dehydrogenase. It reacts with the enzyme in a limited, specific manner with approximately 1 mol of 3′-SBA being incorporated per peptide chain. A determination of the reagent concentration dependence of the rate constant for modification of the enzyme revealed a nonlinear relationship in which an increase in the concentration of 3′-FSBA produced a less-than-proportional increase in the rate constant. Such kinetics are most readily explained in terms of formation of a complex, involving enzyme and 3′-FSBA, prior to the covalent modification. The calculated dissociation constant for that enzyme–3′-FSBA complex (0.100 mM) is comparable in magnitude to the kinetically determined dissociation constants for the complexes of enzyme and ADP (0.015 mM) and of enzyme and the inhibitory DPNH (0.336 mM). The adenosyl moiety plays an essential role in determining the relatively high reactivity of 3′-*p*-fluorosulfonylbenzoyladenosine for glutamate dehydrogenase as demonstrated by the inability of *p*-fluorosulfonylbenzoic acid to cause observable chemical modification of the enzyme. The specificity of 3′-FSBA in modifying glutamate dehydrogenase appears to be considerable, since incubation of the enzyme with a different adenosine analog, in which the fluorosulfonylbenzoyl group is linked to the 5′ (rather than the 3′) position of the ribose ring, does not produce the same pattern of altered kinetic

characteristics (Pal and Colman, unpublished observations).

Insight into the region which has been modified by treatment of glutamate dehydrogenase with 3′-FSBA can be gained by considering both the kinetic parameters of the enzyme which are altered and the ligands which protect against the modification reaction. The *catalytic site* is clearly not modified by 3′-FSBA: during incubation with the reagent, the activity of the enzyme in the absence of regulatory compounds remains constant when TPNH is used as coenzyme. The affinity of the enzyme for its substrates is not significantly changed as indicated by Michaelis constants for α-ketoglutarate, ammonium ion, and DPNH which are essentially the same for modified as for native enzyme. Finally, the participants in the catalytic site: α-ketoglutarate, TPNH, and low levels of DPNH do not provide protection against the kinetic effects of reaction with 3′-FSBA, although it is known that each of these ligands is capable of binding to the enzyme in the absence of other substrates (Rosen *et al.*, 1973; Baker *et al.*, 1962; Cross and Fisher, 1970; Krause *et al.*, 1974).

Another possible locus of attack of 3′-FSBA on glutamate dehydrogenase is the inhibitory GTP site. In the modified enzyme, the kinetically observed dissociation constant for the enzyme–GTP complex is increased about tenfold as compared with the native enzyme, and it might be argued that the covalently bound 3′-SBA group directly interferes with the binding of GTP. On the other hand, it is unlikely that the modified group is actually within the GTP binding site, since the enzyme can still be inhibited 60% by GTP after stoichiometric incorporation of 3′-SBA has occurred; a total loss of inhibition by GTP might have been anticipated if 3′-SBA occupied the GTP binding site. Rather, the altered K_{GTP} is probably an indirect reflection of modification at an adjacent site. The lack of protection of the enzyme against modification when GTP is included in the incubation mixture confirms the postulate that 3′-FSBA does not react within the GTP binding site.

The most reasonable candidates for reaction with 3′-fluorosulfonylbenzoyladenosine on the basis of analogy of the natural ligand to the structure of the reagent are the DPNH and ADP binding sites. Since the enzyme is not inactivated, it is apparent that 3′-FSBA does not attack the first, catalytic DPNH site. However, the characteristic inhibition by high levels of DPNH is completely lost. Indeed, a plot of velocity *vs.* [DPNH] obeys classical Michaelis–Menten kinetics, actually approaching the V_{max} that is only obtained by extrapolation for the native enzyme. It appears that 3′-FSBA reacts within the second DPNH site (thereby preventing the binding of added DPNH), but does not itself inhibit the enzyme. The 3′-FSBA includes only the adenine and ribose moieties in common with the DPNH structure; it may be that the nicotinamide portion of the coenzyme is essential for the inhibitory effect. It is notable that high concentrations of DPNH provide striking protection against the kinetic consequences of modification by 3′-FSBA, perhaps because the reagent and coenzyme utilize a common adenine and ribose binding site. Enzyme incubated with 3′-FSBA in the presence of DPNH still incorporates approximately 1 mol of 3′-sulfonylbenzoyladenosine, despite the fact that it retains full sensitivity to activation by ADP, and is inhibited by high concentrations of DPNH. It is apparent that the inclusion of DPNH during the period of incubation of enzyme and 3′-FSBA must change the specificity of the reagent for the enzyme so that it reacts in a region other than the DPNH binding site.

There remains the possibility that the ADP binding site of glutamate dehydrogenase is also attacked when the enzyme is treated with 3′-FSBA in the absence of ligands. Evidence has been presented that ADP and high levels of DPNH compete for binding to the enzyme (Pantaloni and Dessen, 1969); however, these studies do not distinguish between identical sites for ADP and DPNH and partially or entirely separate sites in which the binding of ADP to its site sterically excludes binding to the inhibitory DPNH site. This paper demonstrates that ADP protects against the kinetic effects of 3′-FSBA modification, as does DPNH; indeed, the modified enzymes prepared in the presence of ADP or DPNH appear very similar in properties. These results underline the intimate relationship between the ADP and DPNH sites. When enzyme is treated with 3′-FSBA in the absence of ligands, the extent of activation by added ADP is markedly reduced. However, the velocity of the modified enzyme is still increased about 30% by the added ADP, implying that the ability of the enzyme to bind ADP has not been abolished. Indeed a normal dissociation constant may be calculated for the enzyme–ADP complex from this reduced enhancement. Moreover, although ADP is a relatively ineffectual activator of the modified enzyme, it is able to displace the inhibitor GTP from the modified as well as the native enzyme. Clearly, the enzyme modified by 3′-FSBA must bind ADP in a manner that is kinetically competitive with GTP. There is no indication that this enzyme can bind DPNH at the inhibitory site.

The simplest explanation of these results is that the GTP, ADP, and inhibitory DPNH sites are not identical, but are at most partially overlapping. For example, the DPNH site may include an adenine site which is shared with ADP, but have unique regions for the binding of the ribosyl, pyrophosphate, and nicotinamide moieties. Adenosine diphosphate may compete with DPNH for the adenine binding site, have its own ribose binding region, and share the phosphate binding region with GTP. Thus it is possible to observe kinetic competition for the ADP–DPNH pair and the ADP–GTP pair, but not the DPNH–GTP pair. It should also be possible to modify an amino acid and affect the binding of only one of a pair. The compound 3′-FSBA appears to react with an amino acid residue within or immediately adjacent to the inhibitory DPNH site, probably in the region where the ribosyl group is normally bound. The 3′-SBA might then sterically occupy both the adenine and ribosyl sites, thus directly blocking the binding of added DPNH. Because ADP and DPNH have a common adenine site, both the activator and the coenzyme would be able to protect the native enzyme against the binding of 3′-FSBA. Once the 3′-SBA is covalently linked to the enzyme, it may necessitate a rearrangement of the characteristic mode of binding of ADP so that the phosphate and ribose moieties bind in their usual locations, but the adenine ring is displaced, thereby causing a loss of the normal ability of ADP to activate the enzyme, but a retention of the ability of ADP to bind to the enzyme and compete with GTP. The bound 3′-SBA probably exerts an indirect role in weakening the binding of GTP at a neighboring site by promoting a local conformational change.

As indicated previously, sulfonyl halides have a relatively broad range of reactivity with amino acid residues in proteins. Studies are currently in progress in this laboratory to identify the tryptic peptide which are labeled with 3′-FSBA in the absence and presence of ADP or DPNH.

References

Anderson, G., and Graves, G. (1973), *Biochemistry 12,* 1895.
Baker, B. R. (1967), Design of Active-Site-Directed Irreversible Enzyme Inhibitors, New York, N.Y., Wiley.
Baker, B. R., Lee, W. W., and Tong, E. (1962), *J. Theor. Biol. 3,* 459-483.
Brunswick, D. J., and Cooperman, B. S. (1971), *Proc. Nat. Acad. Sci. U.S. 68,* 1801.
Clark, C. F., and Yielding, K. L. (1971), *Arch. Biochem. Biophys. 143,* 158.
Colman, R. F., and Frieden, C. (1966a), *J. Biol. Chem. 241,* 3652-3660.
Colman, R. F., and Frieden, C. (1966b), *J. Biol. Chem. 241,* 3661.
Colman, R. F., and Frieden, C. (1966c), *Biochem. Biophys. Res. Commun. 22,* 100.
Cross, D. G., and Fisher, H. F. (1970), *J. Biol. Chem. 245,* 2612-2621.
diPrisco, G. (1971), *Biochemistry 10,* 585.
Fisher, H. F. (1973), *Advan. Enzymol. 39,* 369-417.
Frieden, C. (1959), *J. Biol. Chem. 234,* 809, 815.
Frieden, C. (1963), *J. Biol. Chem. 238,* 3286-3299.
Goldin, B. R., and Frieden, C. (1971), *Biochemistry 10,* 3527-3534.
Goldin, B. R., and Frieden, C. (1972a), *Curr. Top. Cell. Regul. 4,* 77-117.
Goldin, B. R., and Frieden, C. (1972b), *J. Biol. Chem. 247,* 2139-2144.
Hulla, F. W., and Fasold, H. (1972), *Biochemistry 11,* 1056.
Koberstein, R., Krause, J., and Sund, H. (1973), *Eur. J. Biochem. 40,* 543-548.
Koberstein, R., and Sund, H. (1973), *Eur. J. Biochem. 36,* 545-552.
Krause, J., Buhner, M., and Sund, H. (1974), *Eur. J. Biochem. 41,* 593-602.
Olson, J. A., and Anfinsen, C. B. (1952), *J. Biol. Chem. 197,* 67.
Pal, P. K., Wechter, W. J., and Colman, R. F. (1974), Abstracts of the 168th National Meeting of the American Chemical Society, Atlantic City, N.J.
Pantaloni, D., and Dessen, P. (1969), *Eur. J. Biochem. 11,* 510.
Pantaloni, D., and Lécuyer, B. (1973), *Eur. J. Biochem. 40,* 381-401.
Poulos, T. L., and Price, P. A. (1974), *J. Biol. Chem. 249,* 1453.
Price, N. C., and Radda, G. K. (1969), *Biochem. J. 114,* 419.
Rosen, N. L., Bishop, L., Burnett, J. B., Bishop, M. and Colman, R. F. (1973), *J. Biol. Chem. 248,* 7359-7369.
Schaeffer, H. J., and Odin, E. (1966), *J. Med. Chem. 9,* 576.
Smith, E. L., Landon, M., Piszkiewicz, D., Brattin, W. J., Langley, T. J., and Melamed, M. D. (1970), *Proc. Nat. Acad. Sci. U.S. 67,* 724.
Smith, E. L., and Piszkiewicz, D. (1973), *J. Biol. Chem. 248,* 3089-3092.

Structural Proteins of Dogfish Skeletal Muscle†

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ABSTRACT: As part of a study on the evolutionary aspects of control mechanisms, a number of structural muscle components from the Pacific dogfish (*Squalus acanthias*) are described. These include troponin, tropomyosin, actin, and myosin. Troponin (mol wt 108,000) was resolved into its constitutive subunits, represented by a 20,500 mol wt fragment which binds 2 mol of Ca^{2+}/mol with a K_{Diss} of 0.91 μM, and an inhibitory component of 30,000 and a 58,000 component which are necessary for the calcium sensitivity of actomyosin ATPase. Tropomyosin and actin share many properties with their counterparts from higher vertebrates. Proteins similar to parvalbumins, *i.e.*, the low molecular weight calcium-binding proteins widely distributed in fish, amphibians, and mammalian muscle, could be generated from troponin and its calcium-binding subunit by limited proteolysis. The appearance of immunological cross-reactivity and other similar features suggested some identity, but differences in the amino acid analysis exclude the possibility that parvalbumins occur as breakdown products of troponin. The close relationship between parvalbumins and the calcium-binding subunit brings additional evidence that these proteins have arisen through divergent evolution.

An investigation of the structure, function, and interrelationship of calcium-binding proteins in the muscle from the Pacific dogfish (*Squalus acanthias*) was undertaken some time ago as part of a study of the evolutionary aspects of the control of glycogen metabolism.

In this tissue, glycogen breakdown is primarily initiated by the conversion of phosphorylase *b* to *a*, catalyzed by phosphorylase kinase, an enzyme that is totally inactive in the absence of Ca^{2+} ions. Release of calcium from the sarcoplasmic reticulum or other subcellular structures triggers the activation of phosphorylase kinase and therefore, in

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