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Resonance Energy Transfer between the Adenosine 5′-Diphosphate Site of Glutamate Dehydrogenase and a Guanosine 5′-Triphosphate Site Containing a Tyrosine Labeled with 5′-[*p*-(Fluorosulfonyl)benzoyl]-1,N^6-ethenoadenosine†

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ABSTRACT: The fluorescent nucleotide analogue 5′-[*p*-(fluorosulfonyl)benzoyl]-1,N^6-ethenoadenosine (5′-FSBεA) reacts irreversibly with bovine liver glutamate dehydrogenase and modifies one of the natural inhibitory guanosine 5′-triphosphate (GTP) sites [Jacobson, M. A., & Colman, R. F. (1982) *Biochemistry 21*, 2177–2186]. Enzyme with 1.28 mol of 5′-(*p*-sulfonylbenzoyl)-1,N^6-ethenoadenosine/mol of subunit incorporated and exhibiting maximum change in sensitivity to GTP inhibition is now shown by amino acid analysis to contain 0.95 mol of *O*-[(4-carboxyphenyl)sulfonyl]tyrosine (CBS-Tyr) and 0.33 mol of N^{ϵ}-[(4-carboxyphenyl)sulfonyl]-lysine (CBS-Lys), quantitatively accounting for the total incorporation prior to acid hydrolysis. As a function of time of incubation with 5′-FSBεA, the amount of CBS-Tyr formed was directly proportional to the change in GTP inhibition. In contrast, an initial formation of CBS-Lys was observed, followed by relatively little additional CBS-Lys although the percent change in GTP inhibition continued to increase. It was concluded that the tyrosine is an essential residue in the GTP binding site of glutamate dehydrogenase, while the lysine modified is not involved in the inhibitory action of GTP. The nucleotide analogue 2′(3′)-*O*-(2,4,6-trinitrophenyl)adenosine 5′-diphosphate (TNP-ADP) was evaluated for its ability to occupy the adenosine 5′-diphosphate (ADP) activator site and to function as an energy acceptor conjointly with 5′-SBεA covalently bound at the GTP site as the energy donor. TNP-ADP activates native enzyme 2-fold and competes kinetically with ADP. As determined by fluorometric titration, the maximum number of TNP-ADP binding sites on native enzyme was 0.5 mol/mol of subunit in the absence and 1 mol/mol of subunit in the presence of reduced coenzyme. The 5′-SBεA-modified enzyme also binds TNP-ADP: 0.5 mol/mol of subunit in the absence or presence of reduced coenzyme. TNP-ADP competes for binding with ADP to native and 5′-SBεA-modified enzyme, indicating that this nucleotide analogue is a satisfactory fluorescent probe of the ADP site of glutamate dehydrogenase. An energy-transfer efficiency of 0.77 was determined from the decrease in donor fluorescence upon addition of TNP-ADP in the absence of reduced coenzyme to modified enzyme containing 1.23 mol of 5′-SBεA/mol of subunit. A value of 18 Å was calculated as the average distance between the GTP and ADP regulatory sites. This result indicates that the inhibitory GTP and the activatory ADP sites are close but not identical.

The distance between two chromophores on a protein can be determined by fluorescence energy transfer (Förster, 1959) provided that the emission spectrum of the donor chromophore overlaps with the absorption spectrum of the acceptor chromophore and that the two species are sufficiently close to each other (within 80 Å). Studies on a variety of biomolecules and macromolecular assemblies have been carried out by use of energy transfer (Fairclough & Cantor, 1978; Stryer, 1978)

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0006-2960/83/0422-4247$01.50/0

and have produced valuable structural information about the relationship of sites in these systems.

Bovine liver glutamate dehydrogenase [L-glutamate:NAD(P)$^+$ oxidoreductase (deaminating), EC 1.4.1.3] is an allosteric enzyme composed of six identical subunits. The enzyme contains several nucleotide sites per subunit, including a site for the activator ADP,[1] two for the inhibitor GTP, and two for DPNH (one catalytic and one regulatory) (Pantaloni & Dessen, 1969; Goldin & Frieden, 1972; Pal & Colman, 1979; Delabar et al., 1982). The proximity of nucleotide sites on glutamate dehydrogenase has not yet been estimated, although kinetic evidence suggests that there may be some degree of overlap or indirect interaction between the various regulatory sites. For example, ADP competes kinetically with GTP and has been shown to overcome the inhibitory effect produced by the guanine nucleotide (Frieden, 1963). However, the two sites can be independently affected by chemical modification (Colman & Frieden, 1966; Goldin & Frieden, 1971), suggesting that there are two distinguishable nucleotide sites that can influence each other either directly because they partially overlap or indirectly because of conformational changes caused by binding at each site.

In the attempt to identify essential amino acid residues in the regulatory sites of glutamate dehydrogenase, the reactions of several purine nucleotide affinity labels with the enzyme have been studied (Pal et al., 1975a,b; Pal & Colman, 1979; Saradambal et al., 1981). The fluorescent nucleotide analogue 5′-[*p*-(fluorosulfonyl)benzoyl]-1,N^6-ethenoadenosine (5′-FSBεA) has recently been shown to react specifically at one of the GTP sites on glutamate dehydrogenase (Jacobson & Colman, 1982). The extent of incorporation is limited to about 1 mol of reagent/mol of subunit. As compared to native glutamate dehydrogenase, modified enzyme retains full catalytic activity and normal ability to be inhibited by high concentrations of DPNH but exhibits a decreased affinity for GTP and a diminished but not abolished inhibition by saturating concentrations of GTP: the maximum extent of inhibition by GTP is only 70% for modified enzyme as compared with 96% for native enzyme. The modified enzyme binds only 1 mol of GTP/peptide chain rather than the 2 mol of GTP/peptide chain bound by native enzyme, suggesting that the major effect of reaction with 5′-FSBεA is to eliminate one of the natural GTP sites. Although the maximum extent of activation by ADP is only 2.5-fold in the modified enzyme as compared to 4-fold in the native enzyme when assayed with TPNH, the affinity for ADP remains unaltered, indicating that reaction with 5′-FSBεA does not directly affect the ADP activator site. The specific residues modified by 5′-FSBεA were not identified in the initial study. This paper extends the original study by identifying a tyrosine as the essential residue modified by 5′-FSBεA. The introduction of this fluorescent label at a GTP site of glutamate dehydrogenase has now allowed the estimation of a distance by fluorescence energy transfer between this site and 2′(3′)-*O*-(2,4,6-trinitrophenyl)adenosine 5′-diphosphate, as a probe of the ADP activator site.

Experimental Procedures

Materials. Bovine liver glutamate dehydrogenase, purchased as a crystalline suspension in ammonium sulfate from Boehringer Mannheim Corp., was dialyzed for 16 h at 4 °C against two changes of 0.1 M potassium phosphate buffer, pH 7.1. The dialyzed material was centrifuged at 4 °C for 20 min at 15000 rpm to remove precipitated, denatured protein. The enzyme concentration was determined with the value $E^{1\%}_{279}$ = 9.7 (Olson & Anfinsen, 1952); the ratio A_{280}/A_{260} was 1.9. The enzyme was stored in aliquots at −85 °C. A molecular weight of 56100 for identical peptide chains was used in the calculations (Smith et al., 1970).

The compounds 5′-[*p*-(fluorosulfonyl)benzoyl]-1,N^6-ethenoadenosine (5′-FSBεA) and radioactive [2-^{3}H]-5′-FSBεA were synthesized as previously described (Jacobson & Colman, 1982). TNP-ADP was purchased from Molecular Probes. [8-^{14}C]ADP was purchased from New England Nuclear Corp. All coenzymes and purine nucleotides, as well as EDTA and Tris base, were purchased from Sigma Chemical Co. Analytically pure samples of *O*-[(4-carboxyphenyl)sulfonyl]tyrosine and N^ϵ-[(4-carboxyphenyl)sulfonyl]lysine prepared by Saradambal et al. (1981) were made available to us.

Enzymatic Assay. Glutamate dehydrogenase activity was assayed spectrophotometrically at 340 nm by measuring the oxidation of reduced coenzyme at 25 °C in Tris–0.01 M acetate buffer, pH 8 (containing 10 μM EDTA), with a Gilford Model 240 spectrophotometer equipped with an expanded-scale recorder (0–0.1 full scale). For the standard assay, the substrate concentrations used were 5 mM α-ketoglutarate, 50 mM ammonium chloride, and 100 μM DPNH. The total volume of the assay solution was 1.0 mL. When the activity was measured in the presence of a constant concentration of the inhibitor GTP, the nucleotide concentration was 1.0 μM.

Preparation of 5′-SBεA-Modified Enzyme. Glutamate dehydrogenase (1 mg/mL) was incubated with 1.4 mM 5′-FSBεA at 30 °C in 0.01 M sodium barbital buffer (pH 8.0) containing 0.2 M KCl and 10% dimethylformamide. As described previously (Jacobson & Colman, 1982), the loss in GTP inhibition was followed by assaying in the presence of 1 μM GTP. The percent change in GTP inhibition is defined by $(V_t - V_0)/(V_\infty - V_0) \times 100$ where V_t is the enzymatic velocity measured in the presence of GTP at a particular time and V_0 and V_∞ are the velocities measured under the same conditions at time zero and at complete reaction, respectively. At a given extent of reaction, modified enzyme was isolated by the column-centrifuge technique described by Penefsky (1979) with Sephadex G-50, 80 mesh, equilibrated with Tris–0.05 M acetate buffer (pH 8.0) containing 10 mM potassium phosphate and 100 μM EDTA. To prepare modified enzyme exhibiting more than 62% change in sensitivity to GTP inhibition, a second addition of 5′-FSBεA was made after 120 min, so that the final concentration of new reagent was 0.7 mM. Fully modified enzyme exhibiting 100% change in GTP inhibition was isolated, as described above, 1 h after the second addition of reagent was made.

Incorporation of 5′-(p-Sulfonylbenzoyl)-1,N^6-ethenoadenosine by Glutamate Dehydrogenase. The amount of reagent incorporated into the enzyme was determined by either a fluorometric or radiochemical method. Fluorescence measurements were made at 25 °C in a thermostated Hitachi Perkin-Elmer MPF-3 spectrofluorometer, exciting at 310 nm and monitoring emission at 405 nm. The pH of 0.3 mL of modified enzyme, prepared as described above and denatured in 6 M urea, was raised to 12.3 by addition of 5 μL of 3.0 N NaOH. Upon such treatment, a rapid increase of fluorescence intensity was observed followed by a slow loss of fluorescence.

[1] Abbreviations: 5′-FSBεA, 5′-[*p*-(fluorosulfonyl)benzoyl]-1,N^6-ethenoadenosine; 5′-SBεA, 5′-(*p*-sulfonylbenzoyl)-1,N^6-ethenoadenosine; GTP, guanosine 5′-triphosphate; ADP, adenosine 5′-diphosphate; DPNH, reduced diphosphopyridine nucleotide; TPNH, reduced triphosphopyridine nucleotide; TNP-ADP, 2′(3′)-*O*-(2,4,6-trinitrophenyl)adenosine 5′-diphosphate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; CBS-Lys, N^ϵ-[(4-carboxyphenyl)sulfonyl]-lysine; CBS-Tyr, *O*-[(4-carboxyphenyl)sulfonyl]tyrosine.

As has been reported previously (Likos & Colman, 1981), the increase in fluorescence intensity is attributed to the hydrolysis of the ester linkage between the ethenoadenosine and the *p*-(fluorosulfonyl)benzoyl moieties. This cleavage causes the release of free ethenoadenosine, whose quantum yield is 54-fold higher than that of 5′-FSBεA. Extrapolation of the data to time zero was used to obtain the maximum value for the increase in fluorescence, and this measurement was compared to the extrapolated values for 5′-FSBεA standard solutions treated in the same manner.

Alternatively, enzyme was modified as described above by incubation with [2-^{3}H]-5′-FSBεA. After removal of excess reagent by the column-centrifuge method described by Penefsky (1979), the amount of reagent incorporated was determined by measuring the radioactivity in two 100-μL aliquots from the eluate obtained by centrifugation; the aliquots were measured in ACS scintillation liquid (Amersham) with a Packard TriCarb liquid scintillation counter, Model 3330. Protein concentration was determined with the Bio-Rad protein assay, which is based on the method of Bradford (1976). Native glutamate dehydrogenase was used to establish a standard curve. Two successive centrifuge columns were required for complete removal of noncovalently bound reagent.

Identification of Reactive Amino Acid Residues. Modified enzyme of determined incorporation (by the fluorescence technique, as described above) was dialyzed against deionized water for 24 h with repeated changes. After an evaporation to dryness, the modified enzyme was hydrolyzed for 22 h in 6 N HCl at 110 °C. The total hydrolysate was dried, redissolved in water, and applied to an Eastman cellulose thin-layer plate (plastic-backed). Thin-layer electrophoresis was performed with pyridine–acetate buffer, pH 6.4 (pyridine–acetic acid–water, 25:1:250), at 400 V/20 cm for 90 min in order to separate the CBS-derivatized amino acids from all other amino acids. After the plate dried, a small strip of the plate was sprayed with ninhydrin in order to locate CBS-Lys and CBS-Tyr. These residues have a net negative charge at pH 6.4 and migrate in a region between, and well separated from, the neutral and acidic amino acids. This region was removed from the unsprayed plate, extracted 5 times with water, and evaporated to dryness. A standard solution of amino acids containing known amounts of CBS-Lys and CBS-Tyr was treated in an identical manner as described above, and a recovery of 75% for each residue was obtained. A correction for decomposition of CBS-Lys upon acid hydrolysis (15%) was also used as a correction factor in the calculation of CBS residues incorporated into the enzyme. Quantification of CBS-Lys and CBS-Tyr from modified enzyme was accomplished by application of extracts from the electropherogram to a Beckman Model 120C amino acid analyzer after dilution in 0.2 N sodium citrate, pH 2.2. The standard two-column procedure used on the Model 120C was converted to a one-column system as described by Likos & Colman (1981). Under the buffer schedule described, CBS-Lys and CBS-Tyr elute at 168 and 182 min, respectively, as compared to 171 min for tyrosine and 176 min for phenylalanine. The aqueous dimethyl sulfoxide system (Moore, 1968) was used in detecting ninhydrin-positive components. The total amount of protein applied to the thin-layer plate was calculated from amino acid analysis of an aliquot from the hydrolysate and the known amino acid composition of bovine liver glutamate dehydrogenase (Smith et al., 1970).

TNP-ADP Binding Measured by Fluorescence Titration. The binding of TNP-ADP to both native and modified enzymes was measured at 25 °C in Tris–0.05 M acetate buffer (pH 8.0) containing 10 mM potassium phosphate and 100 μM EDTA in a thermostated Perkin-Elmer 650-10S fluorescence spectrophotometer. In the absence of TPNH, samples were excited at 410 nm, and the emission was measured at 545 nm. In the presence of TPNH, samples were excited at 480 nm, and emission was measured at 545 nm to avoid errors arising from absorption by the nucleotide coenzyme. The dissociation constants and number of TNP-ADP binding sites on native and modified enzymes were determined by using the method described by Ehrlich & Colman (1982). The fluorescence of TNP-ADP was measured in the presence, F, and absence, F_0, of enzyme, and the ratio of fluorescence (F/F_0) was used to calculate $[N]_{bound}$ from

$$[N]_{bound} = \frac{[N]_{total}}{Q - 1}\left(\frac{F}{F_0} - 1\right) \quad (1)$$

where $[N]_{total}$ is the total TNP-ADP concentration, $[N]_{bound}$ is the concentration of enzyme-bound TNP-ADP, and Q is the enhancement of TNP-ADP fluorescence for bound ligand. The enhancement factor, Q, was measured by titrating a fixed amount of TNP-ADP (3–4 μM) with increasing amounts of native or 5′-SBεA-modified enzyme in a concentration range of 0.9–7 μM. A double-reciprocal plot of total protein concentration vs. observed fluorescence was extrapolated to infinite protein concentration in order to determine the value of Q. Enhancement factors, Q, for native and 5′-SBεA-modified enzymes in the absence of TPNH (excitation 410 nm) were 5.3 and 6.4, respectively. In the presence of TPNH (excitation 480 nm), Q for native and modified enzymes was measured to be 4.1 and 5.4, respectively. The amount of free TNP-ADP is obtained from the difference of the total TNP-ADP and calculated bound TNP-ADP. The data were analyzed in terms of the Scatchard equation:

$$\frac{\bar{\nu}}{[\text{TNP-ADP}]_{free}} = \frac{n}{K_D} - \frac{\bar{\nu}}{K_D} \quad (2)$$

where $[\text{TNP-ADP}]_{free}$ is the free TNP-ADP concentration, $\bar{\nu}$ is the moles of TNP-ADP bound per enzyme subunit, n is the number of binding sites per subunit, and K_D is the dissociation constant for the enzyme–TNP-ADP complex.

ADP Binding Studies. Binding of [8-^{14}C]ADP to native and modified enzymes in the absence and presence of 10 μM TNP-ADP was measured by an ultrafiltration technique (Colman & Foster, 1970) at 25 °C in Tris–0.05 M acetate buffer (pH 8.0) containing 10 mM potassium phosphate and 100 μM EDTA. An Amicon Model 10-PA ultrafiltration cell was assembled with a PM-10, 25-mm membrane to separate free ligand from enzyme-bound ligand. Both free and total ligand concentrations were determined from the specific activity of [8-^{14}C]ADP with a liquid scintillation counter. The concentration of bound ligand was determined from the difference between the concentrations of total and free ligand.

Resonance Energy-Transfer Measurements. The quantum yield of 5′-SBεA-modified enzyme was measured on a thermostated Hitachi Perkin-Elmer MPF-3 spectrofluorometer at 25 °C equipped with a corrected spectra accessory. A value of 0.01 was obtained from the comparative method of Parker & Reese (1960) by using a quantum yield of 0.70 for a standard solution of quinine sulfate in 0.1 N H_2SO_4 at 25 °C (Scott et al., 1970).

Energy-transfer measurements were carried out by titrating modified enzyme with TNP-ADP and monitoring the decrease in emission at 405 nm (excitation 310 nm). The efficiency of energy transfer is given by

$$E = 1 - Q_{DA}/Q_D \quad (3)$$

where Q_{DA} and Q_D are the quantum yields of 5′-SBεA-modified enzyme in the presence and absence of TNP-ADP, respectively. The wavelength dependence of bound 5′-SBεA excitation and emission is unaffected by the presence of TNP-ADP; thus, the ratio of fluorescence emission at 405 nm (310-nm excitation), F_{DA}/F_D, is proportional to the ratio of the quantum yields and was used to calculate the efficiency, E.

The efficiency of energy transfer was alternatively determined by the sensitized emission of the acceptor TNP-ADP as described by Fairclough & Cantor (1978). The corrected excitation spectrum of the acceptor in the absence and presence of the donor was recorded at a constant emission wavelength of 540 nm (λ_A). The efficiency of energy transfer was calculated by

$$E = \left[\frac{F_{DA}(\lambda_D,\lambda_A)}{F_A(\lambda_D,\lambda_A)} - \frac{A^A_{DA}(\lambda_D)}{A^A_A(\lambda_D)} \right] \frac{A^A_A(\lambda_D)}{A^D_{DA}(\lambda_D)} \quad (4)$$

where $F_{DA}(\lambda_D,\lambda_A)$ and $F_A(\lambda_D,\lambda_A)$ are the magnitudes of the excitation spectra in the region of the donor excitation maximum (310 nm) for acceptor in the presence and absence of donor, respectively, $A^A_{DA}(\lambda_D)$ and $A^A_A(\lambda_D)$ are the absorbances of the acceptor in the presence and absence of donor, respectively, at 310 nm, and $A^D_{DA}(\lambda_D)$ is the absorbance of the donor in the presence of the acceptor at 310 nm.

Results

Incorporation of 5′-FSBεA by Glutamate Dehydrogenase. The extent of covalent incorporation of [2-^{3}H]-5′-FSBεA into glutamate dehydrogenase has previously been shown to be directly proportional to the percent decrease in GTP inhibition and extrapolates to 1.28 mol of 5′-SBεA/mol of enzyme subunit at 100% change in sensitivity to GTP inhibition (Jacobson & Colman, 1982). The incorporation has now also been measured from the fluorescence of ethenoadenosine released from modified enzyme, as described under Experimental Procedures. The incorporation points determined by the fluorescence technique fit on the line generated by the measurements of radioactive reagent incorporated (Figure 1). This result establishes the validity of the fluorescence technique; most of the values of moles of 5′-SBεA per mole of enzyme subunit given in the rest of this paper are based on fluorescence measurements.

Quantification of CBS-Tyr and CBS-Lys in 5′-SBεA-Modified Glutamate Dehydrogenase. Upon acid hydrolysis of modified glutamate dehydrogenase, the ester linkage between the benzoyl and nucleoside moieties of 5′-SBεA is hydrolyzed to yield the products N^{ϵ}-[(4-carboxyphenyl)-sulfonyl]lysine (CBS-Lys) and O-[(4-carboxyphenyl)-sulfonyl]tyrosine (CBS-Tyr). Both modified residues can be identified readily by amino acid analysis as described under Experimental Procedures. Enzyme containing 1.28 mol of 5′-SBεA/mol of subunit and exhibiting 100% change in sensitivity to GTP inhibition was analyzed for content of CBS residues. CBS-Tyr, 0.95 mol, and CBS-Lys, 0.33 mol, were found to account quantitatively for the total incorporation prior to acid hydrolysis, indicating that these are the only amino acid residues in glutamate dehydrogenase modified by 5′-FSBεA. The modification of tyrosine and lysine was determined as a function of time of incubation with 5′-FSBεA and change in GTP inhibition (Figure 2 and Table I). The modification of tyrosine is time dependent; the amount of CBS-Tyr is directly proportional to the change in GTP inhibition. In contrast, there is an initial formation of CBS-Lys early in the incubation of enzyme with 5′-FSBεA, followed by relatively little additional CBS-Lys, although the percent change in GTP inhibition continues to increase. The good correlation between the formation of CBS-Tyr and the percent change in GTP inhibition suggests that the tyrosine modified is in a GTP binding site of glutamate dehydrogenase and that the lysine modified is a nonessential residue not involved in the inhibitory action of GTP.

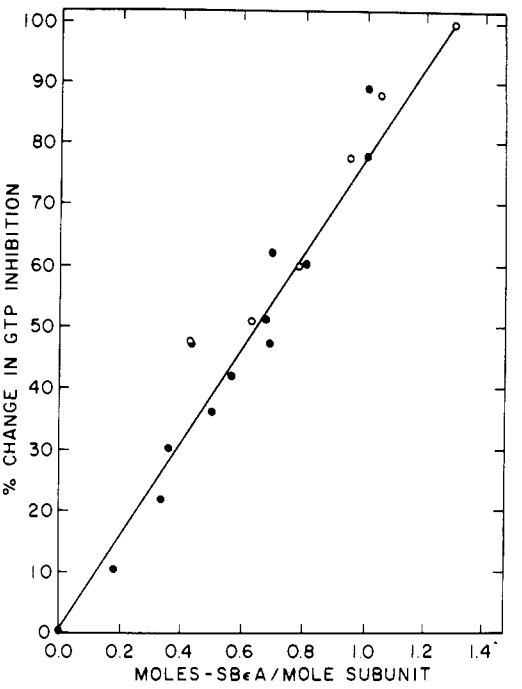

FIGURE 1: Incorporation of 5′-SBεA as a function of percent change in GTP inhibition determined by radioactivity (●) and fluorescence (○). Glutamate dehydrogenase (0.25 mg/mL) was incubated with [2-^{3}H]-5′-FSBεA (1.4 mM); points above 62% change in GTP inhibition were obtained by successive additions of 1.4 and 0.7 mM 5′-FSBεA. Incorporation was determined by either radioactivity or fluorescence as described under Experimental Procedures. The percent change in GTP inhibition is defined by $(V_t - V_0)/(V_\infty - V_0) \times 100$ where V_t is the enzymatic velocity measured in the presence of 1 μM GTP at a particular time and V_0 and V_∞ are the velocities measured under the same conditions at time zero and at complete reaction, respectively.

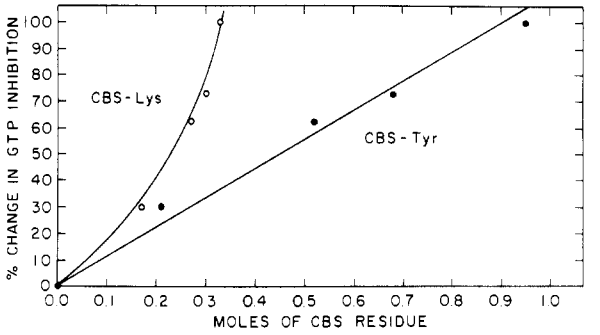

FIGURE 2: Moles of CBS-Lys and CBS-Tyr recovered as a function of percent change in GTP inhibition upon modification of glutamate dehydrogenase with 5′-FSBεA. Incorporation of 5′-FSBεA into glutamate dehydrogenase (1 mg/mL) was measured at various percent changes of GTP inhibition as described under Experimental Procedures. The amounts of modified residues CBS-Lys (○) and CBS-Tyr (●) were determined on an amino acid analyzer, following electrophoretic separation from the other amino acids.

The CBS-Tyr and CBS-Lys were also quantified in enzyme incubated with 5′-FSBεA in the presence of 100 μM GTP and 100 μM DPNH, which protect completely against the change in GTP inhibition. Under these conditions (Table I), a constant incorporation of 0.35 mol of 5′-SBεA/mol of subunit was observed that was independent of time of incubation with

Table I: Quantification of CBS-Tyr and CBS-Lys in Modified Glutamate Dehydrogenase in the Absence and Presence of Protecting Nucleotides[a]

	unprotected enzyme			protected enzyme		
% change in GTP inhibition or equivalent[b]	total incorporation (mol of 5′-SBεA/mol of subunit)	mol of CBS-Tyr/mol of subunit[c]	mol of CBS-Lys/mol of subunit[c]	total incorporation (mol of 5′-SBεA/mol of subunit)	mol of CBS-Tyr/mol of subunit[c]	mol of CBS-Lys/mol of subunit[c]
30	0.38	0.21	0.17	0.35	0.14	0.21
62	0.79	0.52	0.27	0.35	0.14	0.21
73	0.97	0.68	0.29	0.35	0.15	0.20
100	1.28	0.95	0.33	0.35	0.15	0.20

[a] Glutamate dehydrogenase (1 mg/mL) was incubated with 1.4 mM 5′-FSBεA in the absence ("unprotected") or presence of 100 μM GTP and 100 μM DPNH ("protected") at 30 °C in 0.01 M sodium barbital buffer (pH 8.0) containing 0.2 M KCl and 10% dimethylformamide. Modified enzymes above 62% change in GTP inhibition or equivalent were obtained by a second addition of 0.7 mM 5′-FSBεA, as described under Experimental Procedures. [b] Extent of modification is represented in terms of percent change in GTP inhibition. Since no change in GTP inhibition is observed under protecting conditions, "equivalent" implies that "protected enzyme" was prepared by incubation with a concentration of reagent for a duration that would produce the indicated percent change in GTP inhibition in the "unprotected enzyme". [c] Moles of CBS-Tyr or CBS-Lys per mole of subunit is calculated by multiplying the fraction of derivatized amino acid by the total incorporation of 5′-SBεA per mole of subunit as determined by fluorescence.

5′-FSBεA. The total incorporation was decreased in comparison to enzyme modified under the same conditions except for the absence of protecting ligands. The sum of CBS-Tyr and CBS-Lys was found to account for the total incorporation prior to hydrolysis; however, a larger percentage of the total incorporation can be attributed to reaction with lysine as compared with the distribution observed in the unprotected enzyme.

To ascertain whether the residues modified under protected conditions contribute to the incorporation measured in the absence of protecting ligands, the following differential labeling experiment was performed. Glutamate dehydrogenase was modified with nonradioactive 5′-FSBεA in the presence of 100 μM GTP and 100 μM DPNH, as described under Experimental Procedures. Excess reagent and protecting ligands were removed by the column-centrifuge method. Incorporation was determined to be 0.35 mol of 5′-SBεA/mol of subunit as measured by fluorescence after base hydrolysis. This modified enzyme was then incubated with [2-^{3}H]-5′-FSBεA in the absence of ligands and monitored for change in sensitivity to GTP inhibition. Total new incorporation was measured from the radioactivity, as described under Experimental Procedures. The incorporation measured from reaction of [2-^{3}H]-5′-FSBεA with enzyme previously modified with nonradioactive 5′-FSBεA under completely protecting conditions was found to exhibit the same proportionality to the percent decrease in GTP inhibition as enzyme modified directly with [2-^{3}H]-5′-FSBεA in the absence of ligands. At percent changes in GTP inhibition of 30, 53, and 93%, differentially labeled enzyme incorporated 0.41, 0.70, and 1.03 mol of [2-^{3}H]-5′-SBεA/mol of subunit, respectively, which may be compared to 0.41, 0.68, and 1.19 mol of 5′-SBεA/mol of subunit measured directly under nonprotecting conditions. Since prelabeling of glutamate dehydrogenase in the presence of GTP and DPNH does not reduce the subsequent incorporation of 5′-SBεA in the absence of ligands, these results suggest that those residues labeled in the presence of protecting ligands are not the same residues labeled in the absence of protectants and that the 0.35 mol of 5′-SBεA incorporated/mol of subunit in the presence of GTP and DPNH is not included in the total incorporation that occurs in the absence of ligands and that leads to the 100% change in GTP inhibition.

Effect of TNP-ADP on Catalytic Activity of Glutamate Dehydrogenase. Glutamate dehydrogenase is reversibly activated by TNP-ADP as assayed in Tris–0.01 M acetate buffer, pH 8, at 25 °C with either 100 μM DPNH or 100 μM TPNH as the coenzyme. The velocity of the reaction catalyzed by native enzyme as a function of TNP-ADP concentration was measured as shown in Figure 3. The dissociation constant for an enzyme–activator complex has been shown to be numerically equal to the concentration of the activator at which the velocity equals

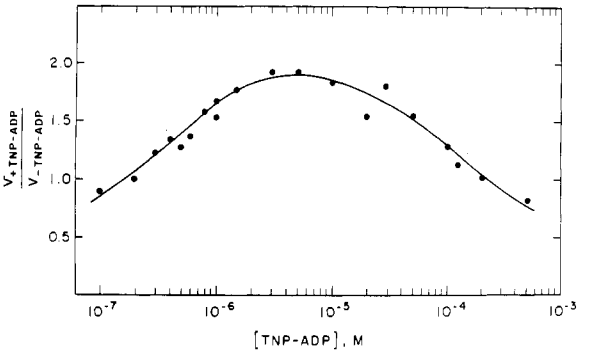

FIGURE 3: Ratio of maximum velocities in the presence and absence of TNP-ADP as a function of TNP-ADP concentration for native enzyme. The velocity was measured as described under Experimental Procedures with 100 μM DPNH as the coenzyme.

$$(1/2)(V + V_A) \quad (5)$$

where V and V_A are the maximum velocities in the absence and presence of saturating concentrations of activator, respectively (Frieden, 1963). As indicated by Figure 3, native enzyme is activated maximally 1.8-fold by TNP-ADP and has a dissociation constant of 0.54 μM as assayed with 100 μM DPNH. For comparison, the affinity of native enzyme for its natural activator ADP is 30 μM with a maximum activation of 2.3-fold as measured with DPNH as coenzyme (Goldin & Frieden, 1972). The dissociation constant for the enzyme–TNP-ADP complex was also measured with 100 μM TPNH as the coenzyme in the enzymatic assay; a dissociation constant of 0.13 μM concomitant with a 2.0-fold activation is observed. This may be compared with a 4-fold activation and a dissociation constant of 7 μM for the native enzyme–ADP complex when TPNH is used as the coenzyme.

To determine if TNP-ADP and ADP compete kinetically for native enzyme, the velocity as a function of ADP concentration was measured in the presence of a constant TNP-ADP concentration, with 100 μM TPNH as the coenzyme in the enzymatic assay. In the presence of 0.4 μM TNP-ADP, an apparent dissociation constant, K_{app}, for the enzyme–ADP complex was measured to be 14 μM, a value 2-fold higher than

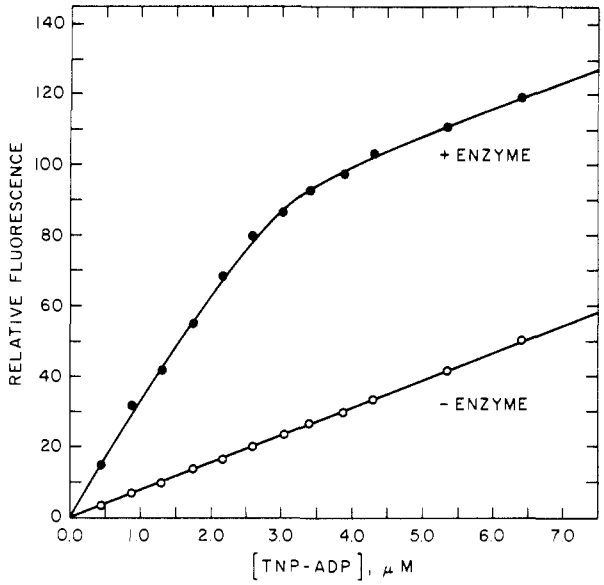

FIGURE 4: Fluorescence titration of native glutamate dehydrogenase with TNP-ADP. Sequential additions of TNP-ADP were made to cuvettes with (●) or without (○) 5 μM enzyme. Titrations were carried out in Tris–0.05 M acetate buffer (pH 8.0) containing 10 mM potassium phosphate and 100 μM EDTA at 25 °C.

the actual K_{ADP} of 7 μM measured in the absence of TNP-ADP. The total extent of activation under these conditions was 3.7-fold. In the presence of the higher constant concentration of 3 μM TNP-ADP, no further activation by ADP was observed. These results suggest that TNP-ADP functions as an activator of glutamate dehydrogenase and competes kinetically with ADP.

The velocity of modified enzyme containing 1.28 mol of 5′-SBεA/mol of subunit and exhibiting 100% of the maximum change in GTP inhibition was measured as a function of TNP-ADP concentration. In contrast to native enzyme, no substantial activation was observed when assayed with either DPNH or TPNH. The modification of glutamate dehydrogenase by 5′-FSBεA has previously been shown to produce an enzyme with a decreased extent of activation by ADP (Jacobson & Colman, 1982). Since TNP-ADP produces less activation than does ADP in the native enzyme, any reduction in the extent of activation upon modification by 5′-FSBεA might make it difficult to detect the activation experimentally.

Titration of TNP-ADP Nucleotide Binding Sites. Titration of binding sites was measured fluorometrically by successive additions of TNP-ADP to the same cuvette in either the presence or the absence of enzyme. The total fluorescence intensity was recorded after each addition. In the presence of 5 μM enzyme (Figure 4), a large fluorescence enhancement is observed concomitant with a blue shift of the TNP-ADP emission maximum from 560 to 545 nm. The nucleotide appears to saturate the enzyme since the slope of TNP-ADP fluorescence in the presence of enzyme approaches that of TNP-ADP in buffer alone above 5 μM. The concentration of bound TNP-ADP is calculated from eq 1 and used to determine $\bar{\nu}$ in eq 2. The dissociation constants for enzyme–TNP-ADP complexes and number of binding sites in the absence and presence of TPNH were evaluated by Scatchard analysis from eq 2.

In the absence of TPNH (Figure 5A), native enzyme exhibits a nonlinear Scatchard plot that can be described by an apparent cooperativity among subunits. A dissociation constant of 0.30 μM is determined from the limiting slope of the curve at values of $\bar{\nu} \geq 0.25$. The maximum number of sites

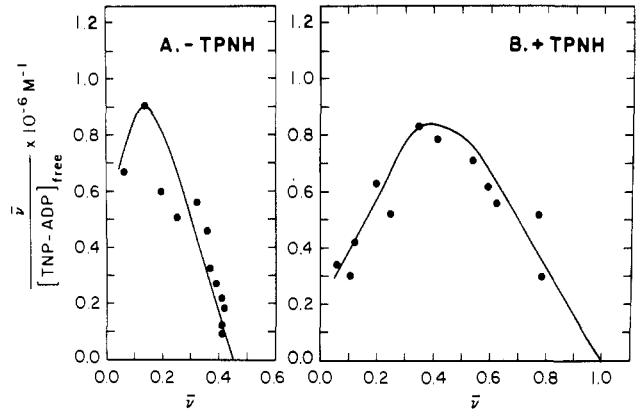

FIGURE 5: Binding of TNP-ADP to native glutamate dehydrogenase. (A) Native enzyme (5 μM) in the absence of TPNH; (B) native enzyme (5 μM) in the presence of 100 μM TPNH. Buffer conditions are as described in Figure 4.

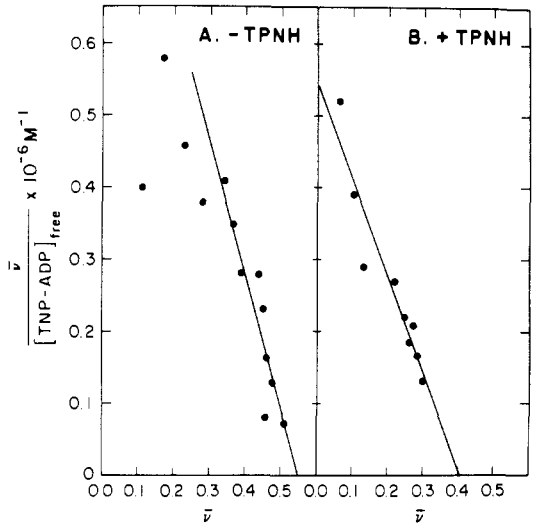

FIGURE 6: Binding of TNP-ADP to 5′-SBεA-modified enzyme. Modified enzyme (5 μM) containing 1.28 mol of 5′-SBεA/mol of subunit in (A) the absence and (B) presence of 100 μM TPNH. Buffer conditions are as described in Figure 4.

(n) is 0.46 mol of TNP-ADP/mol of enzyme subunit. In the presence of TPNH (Figure 5B), a nonlinear Scatchard plot is also observed; however, the number of TNP-ADP binding sites is 1.0 mol/mol of enzyme subunit. From the limiting slope of the curve at $\bar{\nu} \geq 0.5$, a dissociation constant of 0.60 μM was determined.

Glutamate dehydrogenase containing 1.28 mol of 5′-SBεA/mol of enzyme subunit was titrated as described above with successive additions of TNP-ADP in both the absence and presence of TPNH. An enhancement in fluorescence and a shift in the emission maximum similar to that for native enzyme were observed for modified enzyme. From a Scatchard plot analysis of the data in the absence of TPNH (Figure 6A), a dissociation constant for the 5′-SBεA-modified enzyme–TNP-ADP complex was calculated to be 0.52 μM, with a maximum of 0.54 mol of TNP-ADP/mol of enzyme subunit. In the presence of TPNH, in contrast to native enzyme, only 0.40 mol of TNP-ADP binds to modified enzyme with a dissociation constant of 0.75 μM (Figure 6B). It is clear that 5′-SBεA-modified enzyme binds TNP-ADP, even though no activation of this altered enzyme by TNP-ADP was detected.

Binding of ADP by Native and Modified Enzymes in the Presence of TNP-ADP. It appears that TNP-ADP competes

kinetically with ADP for native enzyme. To ascertain more directly whether TNP-ADP competes with ADP for binding to both native and modified enzymes, the binding of [8-^{14}C]ADP was determined in the presence of saturating concentrations of TNP-ADP by an ultrafiltration technique as described under Experimental Procedures. Measurements were made on 5 μM enzyme at 25 °C in Tris–0.05 M acetate buffer (pH 8.0) containing 10 mM potassium phosphate and 100 μM EDTA. Total concentration of [8-^{14}C]ADP used in the binding mixture containing native or modified enzyme was 5 μM. In the absence of TNP-ADP, native enzyme binds 0.62 mol of ADP/mol of enzyme subunit, a result consistent with the previously measured binding constant for ADP (Frieden & Colman, 1967). When the binding is carried out in the presence of 10 μM TNP-ADP but under otherwise identical conditions, a marked decrease in ADP binding is observed to 0.043 mol of ADP/mol of enzyme subunit. For modified enzyme containing 1.28 mol of 5′-SBϵA/mol of enzyme subunit, only 0.016 mol of ADP/mol of enzyme subunit is bound in the presence of 10 μM TNP-ADP, in comparison to 0.45 mol of ADP/mol of enzyme bound in the absence of TNP-ADP. These results strongly suggest that TNP-ADP competes for binding with ADP on both native and modified enzymes and that, therefore, TNP-ADP can serve as a fluorescent probe of the ADP binding site of glutamate dehydrogenase.

Measurement of Energy Transfer between Nucleotide Sites. The distance between a GTP inhibitory site covalently labeled with 5′-SBϵA and an ADP activatory site containing reversibly bound TNP-ADP was determined in the absence of TPNH by using the Förster (1959) theory of energy transfer. The energy-transfer efficiency, E, is related to the distance between the donor and acceptor sites, R, by

$$R = R_0(E^{-1} - 1)^{1/6} \quad (6)$$

where R_0 is the "critical transfer distance" at which E is 50%. The value of R_0 is calculated from specific properties of the energy donor and acceptor by

$$R_0 = (9.79 \times 10^3)(JK^2Q_Dn^{-4})^{1/6}\ \text{Å} \quad (7)$$

In eq 7, n is the refractive index of the medium, Q_D is the quantum yield of the donor in the absence of acceptor, K^2 is an orientation factor dependent on the relative orientations of the donor and acceptor transition dipoles, and J is the spectral overlap integral of donor fluorescence and acceptor absorption. In the calculation of R_0, the refractive index of water, 1.4, was used for n. The quantum yield of 0.01 for 5′-SBϵA-modified enzyme was determined from the corrected emission spectrum as described under Experimental Procedures. The orientation factor, K^2, was assumed to be $^2/_3$. This is the calculated value for donor and acceptor dipoles rotating rapidly compared to the fluorescence lifetime of the donor (Förster, 1959). Since the absorption spectrum of TNP-ADP overlaps substantially with the fluorescence spectrum of 5′-SBϵA-modified enzyme, the 5′-SBϵA and TNP-ADP constitute a suitable donor–acceptor pair for distance measurements based on fluorescence energy transfer. The spectral overlap, J, is calculated from Figure 7 by using the overlap integral

$$J = \frac{\sum F_D(\lambda)\epsilon_A(\lambda)\lambda^4\Delta\lambda}{\sum F_D(\lambda)\Delta\lambda} \quad (8)$$

and integrating by 5-nm intervals. In eq 8, $F_D(\lambda)$ is the corrected emission of the donor, 5′-SBϵA-modified enzyme, and $\epsilon_A(\lambda)$ is the extinction coefficient of the acceptor, TNP-ADP. A value of 7.19×10^{-14} M^{-1} cm^3 was calculated for J. From eq 6, the calculated value of R_0 is 22 Å.

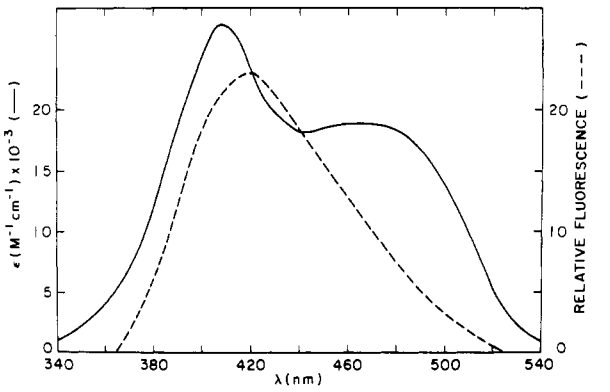

FIGURE 7: Spectral overlap of corrected fluorescence emission of 5′-SBϵA-modified enzyme excited at 310 nm (– –) with the extinction coefficient (ϵ) of TNP-ADP (—) in Tris–0.05 M acetate buffer (pH 8.0) containing 10 mM potassium phosphate and 100 μM EDTA.

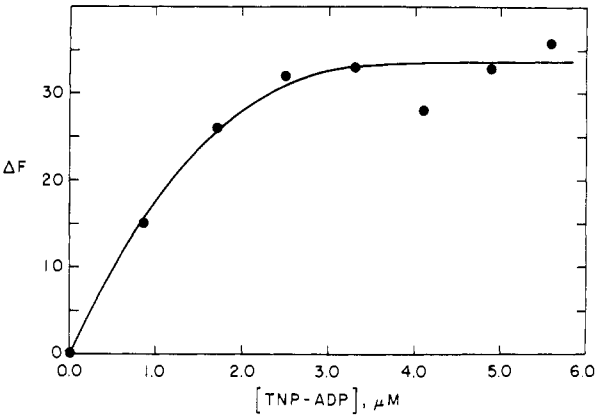

FIGURE 8: Plot of quenching of 5′-SBϵA-modified enzyme fluorescence emission (405 nm, 310-nm excitation), ΔF, as a function of total TNP-ADP concentration. The titration of 5 μM modified enzyme containing 1.14 mol of 5′-SBϵA/mol of subunit was carried out at 25 °C in Tris–0.05 M acetate buffer (pH 8.0) containing 10 mM potassium phosphate and 100 μM EDTA. ΔF is calculated from the difference between the fluorescence observed in the absence and presence of varying concentrations of TNP-ADP. Fluorescence in the absence of TNP-ADP was 56 relative fluorescence units.

The quenching of donor fluorescence from 5′-SBϵA bound at a GTP site on glutamate dehydrogenase upon the reversible binding of TNP-ADP to an activating site was used to calculate the energy-transfer efficiency between these two sites. The decrease in fluorescence at 405 nm (excitation 310 nm) upon titration of 5′-SBϵA-modified enzyme with TNP-ADP is shown in Figure 8. Titration of 5′-SBϵA-modified enzyme with the nonfluorescent natural activator, ADP, in the concentration range of 0–230 μM resulted in no quenching of donor fluorescence at 405 nm. This provides evidence that the decrease in fluorescence is the result of energy transfer between the 5′-SBϵA and TNP-ADP chromophores rather than of a conformational change in the protein induced by the acceptor.

The efficiency of energy transfer was calculated from eq 3 for each point in a titration curve generated over the range of 2.5–6.0 μM TNP-ADP. The observed efficiency was corrected for the fraction of maximum TNP-ADP sites occupied at a given TNP-ADP concentration by using the Scatchard plot in Figure 6A and

$$E_c = E_{obsd}/f_A \quad (9)$$

where E_c and E_{obsd} are the corrected and observed efficiencies of energy transfer, respectively, and f_A is the fraction of the maximum sites occupied by the acceptor, TNP-ADP.

Table II: Energy-Transfer Measurement as a Function of Extent of Modification[a]

mol of 5′-SBϵA/mol of subunit	energy-transfer efficiency	R (Å)
Unprotected Enzyme		
0.41	0.51	2.18 ± 0.6
0.85	0.42	23.2 ± 0.2
1.14	0.68	19.5 ± 1.0
1.23	0.77	17.9 ± 1.4
Protected Enzyme		
0.35	0.53	21.8 ± 0.5

[a] 5′-SBϵA-modified enzyme (5 μM) was titrated with TNP-ADP in Tris–0.05 M acetate buffer (pH 8.0) containing 10 mM potassium phosphate and 100 μM EDTA as described under Experimental Procedures. The efficiency of energy transfer as measured by quenching of donor fluorescence was corrected for the fraction of maximum TNP-ADP sites occupied at a given TNP-ADP concentration as described under Results. The distance between sites, R, was calculated from eq 6 with R_0 = 22 Å.

Throughout the range of 2.5–6.0 μM TNP-ADP (corresponding to $\bar{\nu}$ = 0.35–0.54), the corrected efficiency of energy transfer was constant.

In Table II, the efficiencies of energy transfer and distances, R, for preparations of 5′-SBϵA-modified enzyme with different extents of donor incorporation are given. The efficiency is observed to increase as a function of extent of modification. The distance between the GTP and ADP sites is calculated from the efficiencies and eq 6. Modified enzyme containing 1.23 mol of 5′-SBϵA/mol of subunit and exhibiting 100% change in GTP inhibition yielded an efficiency of energy transfer of 0.77. From this efficiency, a value of 18 Å was determined as the average distance between the two nucleotide sites on glutamate dehydrogenase. Modified enzymes with less extensive modification yield distances that are 2–3 Å larger in comparison to fully modified enzyme. The probability of a donor molecule being closer to an acceptor molecule may be higher in modified enzymes of greater extents of 5′-SBϵA incorporation. In this case, a higher efficiency of transfer is observed that is reflected in a smaller distance, R.

The efficiency of energy transfer was also measured by using the sensitization of acceptor fluorescence by recording the excitation spectra of enzyme-bound TNP-ADP in the absence and presence of the 5′-SBϵA donor. An efficiency of 0.81 was calculated, from which a value of 17.3 Å was determined for the average distance between the GTP and ADP sites. The value obtained by the method of sensitized emission is in good agreement with the 18-Å value obtained by donor quenching and provides additional evidence that the quenching of donor fluorescence by the acceptor is due to energy transfer.

Enzyme modified in the presence of protecting ligands, 100 μM GTP and 100 μM DPNH, containing 0.35 mol of 5′-SBϵA/mol of subunit was also examined for the ability of energy transfer between covalently bound 5′-SBϵA and TNP-ADP. The fluorescence at 405 nm (310-nm excitation) is also quenched in the presence of TNP-ADP. An efficiency of 0.53 was determined, which results in a distance of 22 Å. The above explanation for modified enzymes of less extensive modification can also be applied here. However, this value of 22 Å is a distance between an unidentified site not involved in GTP inhibition and the TNP-ADP site.

Discussion

The reaction of 5′-FSBϵA with glutamate dehydrogenase has been described previously (Jacobson & Colman, 1982). The fluorescent nucleotide analogue was found to exhibit the characteristics of an affinity label of a GTP inhibitory site on the enzyme; however, the specific amino acid modified in this site by 5′-FSBϵA was not identified. The sulfonyl fluoride moiety of 5′-FSBϵA has the potential for sulfonating cysteine, serine, lysine, tyrosine, histidine, and threonine residues (Paulos & Price, 1974). The observation has been made with 5′-FSBϵA (Likos & Colman, 1981) and with other fluorosulfonyl derivatives of adenosine and guanosine (Annamalai & Colman, 1981; Tomich et al., 1981) that addition of dithiothreitol reverses the reaction of the analogue with certain enzymes. These results have been interpreted to indicate the involvement of a thiosulfonate linkage between the reagent and a cysteine at the modification site. As previously shown (Jacobson & Colman, 1982), dithiothreitol had no effect on the kinetics of the reaction or on the incorporation of 5′-FSBϵA by glutamate dehydrogenase, indicating that cysteine is not the target of modification by 5′-FSBϵA. By use of amino acid analysis, both tyrosine and lysine were identified as the amino acids modified by 5′-FSBϵA in glutamate dehydrogenase. The incorporation measured prior to hydrolysis of modified enzyme is quantitatively accounted for by the sum of CBS-Tyr and CBS-Lys detected by amino acid analysis, suggesting that there are no other amino acids modified by 5′-FSBϵA. The time-dependent modification of tyrosine was found to be directly proportional to the percent change in GTP inhibition, extrapolating to about 1 mol of modified tyrosine/mol of subunit at 100% change in GTP inhibition. In contrast, the extent of modification of lysine is insufficient by itself to account for the percent change in GTP inhibition: at 100% change in GTP inhibition, only 0.33 mol of CBS-Lys/mol of subunit is observed. Furthermore, the extent of lysine modification does not correlate with the change in GTP inhibition. The tyrosine modified by 5′-FSBϵA is thus believed to be a critical residue in the GTP binding site of glutamate dehydrogenase. The implication of tyrosine as the site of modification is not the first report of the involvement of tyrosine in the GTP inhibitory site of glutamate dehydrogenase. Earlier studies have demonstrated such a role for tyrosine (Price & Radda, 1969; Piszkiewicz et al., 1971; Smith & Piszkiewicz, 1973). Nitration of tyrosine by tetranitromethane had no effect on the catalytic activity of the enzyme; however, the inhibition afforded by GTP was dramatically decreased. The peptide containing the modified tyrosine has been isolated, and the residue was identified as tyrosine-407, according to the numbering of the glutamate dehydrogenase sequence (Julliard & Smith, 1979). It is possible that the tyrosine modified by 5′-FSBϵA may be the same tyrosine modified by tetranitromethane since the effect of modification on the kinetic properties of the enzyme are similar. Studies are in progress to isolate the peptide labeled with 5′-FSBϵA.

The spectral properties of the ADP analogue 2′(3′)-*O*-(2,4,6-trinitrophenyl)adenosine 5′-diphosphate are suitable for use as an energy acceptor conjointly with 5′-SBϵA-modified enzyme as a donor in an energy-transfer measurement; and the relationship of TNP-ADP to the ADP site on glutamate dehydrogenase has been established under Results. Native enzyme is activated 2-fold by TNP-ADP, and through kinetic and direct-binding measurements, competition with the natural activator, ADP, was demonstrated. The dissociation constant for native enzyme–TNP-ADP complex was determined kinetically to be 55-fold tighter than the dissociation constant determined for enzyme–ADP complex when assayed with either DPNH or TPNH.

The relationship between the structure of purine nucleotides and their effects on the activity of glutamate dehydrogenase has been discussed by Lascu et al. (1977). ADP analogues with modified terminal phosphate groups such as 5′-adenylyl methylenediphosphate and 5′-adenylyl imidodiphosphate activated the enzyme, although to a lesser extent than the natural activator, ADP. Structural analogues of ADP with modification in the purine moiety inhibited the enzyme, suggesting that the inhibitory GTP site is not very specific for the structural integrity of the nucleotide effector. In contrast, the ADP activating site appears to require an intact adenosine diphosphate to be an activator. TNP-ADP has now been demonstrated to be an activator for glutamate dehydrogenase. It can be postulated that the 2′ and 3′ positions of the ribose ring are not critical for recognition of the nucleotide as an activator by the enzyme. The trinitrophenyl groups of TNP-ADP are negatively charged at neutral pH (Hiratsuka & Uchida, 1973). The tighter binding of TNP-ADP (as compared to ADP) by native enzyme may be explained by an increased attraction between the enzyme and the additional negative charge resulting from the ionizable trinitrophenyl group on the analogue.

The fluorescent enhancement and shift in fluorescence emission from 560 to 545 nm upon the binding of TNP-ADP to glutamate dehydrogenase is indicative of an interaction between the chromophoric nucleotide and an environment similar to that observed for TNP-ADP alone in methanol (dielectric constant = 32). In methanol, a blue shift in the emission maximum to 543 nm concomitant with a 7-fold increase in the quantum yield was observed for TNP-ADP in comparison to that in water (Moczydlowski & Fortes, 1981).

The maximum number of TNP-ADP binding sites was determined by fluorometric titration to be about 0.5 mol/mol of subunit in the absence of TPNH and 1 mol/mol of subunit in the presence of TPNH. In both cases, the Scatchard plots exhibited nonlinearity, indicating apparent cooperativity among subunits. An apparent cooperativity among subunits has previously been observed for the inhibitor GTP (Frieden & Colman, 1967) and for zinc (Colman & Foster, 1970). However, in contrast to the present observations for the binding of TNP-ADP, no cooperativity or half-site reactivity was observed for the binding of ADP to glutamate dehydrogenase (Frieden & Colman, 1967). Additionally, the binding of TNP-ADP to three of the six subunits appears to prevent binding of ADP to all of the subunits. These results suggest a definite interaction among the subunits of glutamate dehydrogenase. Further evidence for interacting subunits has been provided by chemical-modification studies. The adenosine analogue 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine covalently modifies half of the subunits, causing total elimination of DPNH inhibition of all the subunits. Trinitrophenylation of lysine-428[2] on half of the subunits has also been shown to cause total loss of DPNH inhibition (Coffee et al., 1971; Goldin & Frieden, 1971). Electron micrographs and low-angle X-ray and cross-linking studies (Hucho et al., 1975; Sund et al., 1975; Eisenberg et al., 1976) suggest that the six subunits of glutamate dehydrogenase are arranged in two groups of three, in the form of two triangles layered on one another. The half-sites reactivity observed may in part be a reflection of the structural arrangement of the enzyme constituting two different binding domains having different reactivities to regulatory compounds.

[2] This is lysine-423 according to the sequence of numbering of Julliard & Smith (1979).

Enzyme modified by 5′-[*p*-(fluorosulfonyl)benzoyl]-1,N^6-ethenoadenosine also binds TNP-ADP. In the absence and presence of TPNH, linear Scatchard plots resulted that extrapolate to about 0.5 mol of TNP-ADP bound/mol of subunit. It appears that half of the TNP-ADP binding sites measured in the presence of TPNH on native enzyme are eliminated as the result of modification with 5′-FSBεA. In addition, a loss in the apparent cooperativity among subunits for TNP-ADP binding results from the modification. Nevertheless, TNP-ADP still competes with ADP for binding to the modified enzyme. It is concluded that TNP-ADP is a satisfactory probe of the ADP site on both native and 5′-SBεA-modified enzyme and can reasonably be employed as an energy acceptor in energy-transfer measurements.

The distance between a GTP inhibitory site covalently labeled with 5′-SBεA and an ADP activating site containing reversibly bound TNP-ADP was determined by fluorescence energy transfer from the theory of Förster (1959). The quantum yield of 5′-SBεA-modified enzyme was measured as 0.01 and used in the calculation of R_0. For comparison, the quantum yield of 5′-FSBεA in phosphate buffer, pH 7.0, was reported to be approximately 0.01 (Likos & Colman, 1981). It was postulated that 5′-FSBεA in aqueous solution exists in a stacked conformation in which the purine base and the benzoyl group interact to cause internal quenching of the ethenoadenosine fluorescence. This stacking was thought to account for the low quantum yield in comparison to that of 0.54 reported for ethenoadenosine (Secrist et al., 1972). Since the same value of 0.01 was measured for 5′-SBεA covalently bound to glutamate dehydrogenase, it is likely that the fluorescent probe exists in the same stacked conformation on the enzyme as it does in aqueous solution.

The accuracy of a distance measured by fluorescence energy transfer is limited by the uncertainty in the orientation factor K^2 used in calculating R_0. Theoretically, K^2 may assume any value between 0 and 4, depending on the relative orientations of the donor and acceptor transition dipoles (Fairclough & Cantor, 1978). If both donor and acceptor dipoles have complete rotational freedom and can assume all orientations during the lifetime of the donor in the excited state, a value of $^2/_3$ for K^2 is used in the calculation of R_0 (Förster, 1959). From the fluorescence polarization of the energy donor, the upper and lower limits for K^2 can be calculated and used to determine a range of R_0's (Dale et al., 1979). However, the determination of the fluorescence polarization of covalently bound 5′-SBεA was not available, and thus, the assumption of $K^2 = {}^2/_3$ was utilized in the calculations of the distance between nucleotide sites on glutamate dehydrogenase. Since at least one member of the energy-transfer pair may have some degree of rotational mobility, the uncertainty in the value of K^2 has been postulated to produce an uncertainity not greater than 10% in the distances measured by energy transfer (Matsumoto & Hammes, 1975).

A distance of 18 Å between the GTP and ADP sites was calculated from the quenching of donor fluorescence. This value must be a measure of the distance between the purine ring of the 5′-SBεA (at the GTP binding site) and the TNP moiety linked to the ribose moiety of TNP-ADP (at the ADP site). Because of the difference in donor–acceptor stoichiometry (about 1 mol of 5′-SBεA/0.5 mol of TNP-ADP), this value is an average distance between these two regulatory sites. A number of models can be postulated in the attempt to interpret the 18-Å distance in terms of the relative location of the one acceptor and two donor molecules. If it is assumed that two donor molecules are equally quenched by an acceptor

molecule, then the efficiency of energy transfer is the same as the observed efficiency, and the resultant distance, R, is the average distance between any donor and acceptor molecule. Alternatively, if one donor is much closer to the acceptor and is entirely responsible for the observed decrease in the donor fluorescence, then the actual efficiency of energy transfer would be twice the measured apparent efficiency (Matsumoto & Hammes, 1975; Cardon & Hammes, 1982). Since the measured efficiency of energy transfer between 5′-SBεA and TNP-ADP on glutamate dehydrogenase is greater than 0.5, the experimental data must reflect some contribution to the energy transfer to TNP-ADP from all of the donors; it is possible, however, that the contributions of the donors are not equal. Since glutamate dehydrogenase is a hexameric protein, the ratio of about 1 donor/0.5 acceptor per subunit really represents 5′-SBεA bound to six subunits with TNP-ADP bound to only three of those subunits. It seems most likely that the major contribution to energy transfer occurs between donor and acceptor molecules located on the same subunit. Therefore, the average value of 18 Å is probably an upper limit of the distance between the GTP and ADP sites within one subunit.

A small decrease in the distance between donor and acceptor was observed as a function of extent of 5′-SBεA modification. Because of the faster rate of lysine modification by 5′-FSBεA in comparison to modification of the tyrosine on glutamate dehydrogenase, modified enzyme containing fewer groups incorporated has a greater percentage of the total incorporation attributable to lysine than do modified enzymes with greater extents of modification. If the donor-labeled lysine contributed significantly to the distance measurement between the tyrosine and the ADP site, a much different distance value should be obtained from modified enzyme containing less incorporation than in more fully modified enzyme. However, the 2–3-Å difference as a function of extent of 5′-SBεA modification does not appear sufficient to suggest that the small percentage of nonessential lysine modified in addition to the 1 mol of tyrosine influences significantly the distance measurement. It may, rather, reflect the decreased probability at low extents of 5′-SBεA incorporation of having donor and acceptor molecules bound to the same subunit.

The energy-transfer results suggest clearly that the GTP and ADP regulatory sites are not identical. This conclusion is consistent with the results in which the two sites were demonstrated to be independently affected by acetylation (Colman & Frieden, 1966) and trinitrophenylation (Goldin & Frieden, 1971). However, it can be concluded that the GTP and ADP sites are relatively close to one another, which may explain the observed kinetic competition (Frieden, 1963) between these two nucleotides for glutamate dehydrogenase.

Registry No. 5′-FSBεA, 76021-83-5; ADP, 58-64-0; GTP, 86-01-1; TNP-ADP, 77450-67-0; L-tyrosine, 60-18-4; EC 1.4.1.3, 9029-12-3.

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Conformational Changes of Adrenocorticotropin Peptides upon Interaction with Lipid Membranes Revealed by Infrared Attenuated Total Reflection Spectroscopy†

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ABSTRACT: Infrared attenuated total reflection (IR-ATR) spectroscopy was used to study conformational and topological aspects of the interaction between two adrenocorticotropin fragments and dioleoylphosphatidylcholine membranes. Corticotropin-(1–10)-decapeptide, $ACTH_{1-10}$, was found to exist as a rigid antiparallel pleated sheet structure in dry membranes. In aqueous environment, it completely escaped from the lipid. This dominant preference for the aqueous phase is a possible explanation for the very low biological potency of $ACTH_{1-10}$ in some assays. On the other hand, the very potent corticotropin-(1–24)-tetracosapeptide, $ACTH_{1-24}$, was firmly incorporated into dry and wet membranes. Aqueous environment even promoted the peptide–lipid interaction. Under these latter conditions, part of the molecule entered the bilayer and adopted a helical structure with the axis oriented perpendicularly to the bilayer plane. Contact of a 0.1 mM solution of $ACTH_{1-24}$ in liquid deuterium oxide with the pure lipid membrane system resulted in measurable adsorption of the peptide to the membrane with the same conformational and topological characteristics as described above (perpendicularly oriented helix entering the bilayer). The helical part of the $ACTH_{1-24}$ molecule entering the bilayer was the quite hydrophobic N-terminal decapeptide unit ("message" segment). The adjacent hydrophilic C-terminal tetradecapeptide unit ("address" segment) remained on the membrane surface. As the message region is essential for triggering corticotropin receptors, its intrusion into the membrane and its adoption of an oriented, helical conformation may facilitate receptor stimulation. Our experiments clearly demonstrate that the observed specific membrane interaction critically depended on the presence of the address segment. This could explain the potentiating effect of the "address" in pharmacological experiments.

Adrenocorticotrophic hormone (adrenocorticotropin, corticotropin, ACTH;[1] Table I) is a linear nonatriacontapeptide elaborated in the anterior lobe of the pituitary gland and in certain regions of the brain [for recent reviews, see Schwyzer (1977, 1982)]. ACTH acts on the adrenal cortex, the skin, and the central nervous system, causing steroidogenesis, melanophore darkening, and behavioral changes, respectively. It exerts its effects through saturable and reversible interactions with specific receptors contained in the outer membranes of its target cells [see Schulster & Schwyzer (1980)].

Synthetic N-terminal segments of ACTH are found to be responsible for the hormonal activity and to have specific receptor binding properties (Schwyzer et al., 1960; Lang et al., 1974). Thus, adrenocorticotropin-(1–24)-tetracosapeptide ($ACTH_{1-24}$; Schwyzer & Kappeler, 1963) is a fully potent full agonist of established therapeutic value. Adrenocorticotropin-(1–10)-decapeptide ($ACTH_{1-10}$) is also a full agonist, but with very low potency in adrenal cells (10^{-6}–10^{-5} × [$ACTH_{1-24}$]; Schwyzer et al., 1971), low potency in melanophores (about 10^{-2} × [$ACTH_{1-24}$]; Eberle & Schwyzer, 1976), and high potency (equal to [$ACTH_{1-24}$]; de Wied et al., 1975) in a behavioral assay. Adrenocorticotropin-(11–24)-tetradecapeptide is an antagonist for steroidogenesis (Seelig et al., 1971) without any agonist properties in this and the other two assays. It is concluded that segment 1–10 of $ACTH_{1-24}$ carries the message for triggering the receptor response and that segment 11–24 modulates the potency of the message segment with respect to different receptors (address function) [see Schwyzer (1977)]. The molecular mechanism responsible for the drastic potentiation of the message by the address in the adrenal cell response is unknown: Enhancement of specific affinity for the receptor through additional, specific binding energy between address and re-

Table I: Primary Structure of Adrenocorticotrophic Hormone of Human Origin at Neutral pH

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(+) Ser-Tyr-Ser-Met-Glu(–)-His-Phe-Arg(+)-Trp-Gly-Lys(+)-Pro-
     1               5                      10
Val-Gly-Lys(+)-Lys(+)-Arg(+)-Arg(+)-Pro-Val-Lys(+)-Val-Tyr-
      15                             20
Pro-Asn-Gly-Ala-Glu(–)-Asp(–)-Glu(–)-Ser-Ala-Glu(–)-Ala-Phe-Pro-
24  25                         30                         35
Leu-Glu(–)-Phe(–)
           39
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[1] Abbreviations: ACTH, adrenocorticotrophic hormone; $ACTH_{n-m}$, synthetic ACTH peptides comprising the amino acid residues *n–m* of the natural sequence; IR-ATR, infrared attenuated total reflection.

0006-2960/83/0422-4257$01.50/0