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Distance Relationships between the Catalytic Site Labeled with 4-(Iodoacetamido)salicylic Acid and Regulatory Sites of Glutamate Dehydrogenase†

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ABSTRACT: The distance between the catalytic site on bovine liver glutamate dehydrogenase labeled with 4-(iodoacetamido)salicylic acid (ISA) and the adenosine 5′-diphosphate (ADP) activatory site occupied by the analogue 2′,3′-*O*-(2,4,6-trinitrocyclohexadienylidene)adenosine 5′-diphosphate (TNP-ADP) was evaluated by energy transfer. Native enzyme and enzyme containing about 1 mol of acetamidosalicylate/mol of subunit bind about 0.5 mol of TNP-ADP/mol of subunit, and TNP-ADP competes for binding with ADP to native and modified enzyme, indicating that the analogue is a satisfactory probe of the ADP site. From the quenching of acetamidosalicylate donor fluorescence upon addition of TNP-ADP, an average distance of 33 Å was determined between the catalytic and ADP sites. The fluorescent nucleotide analogue 5′-[*p*-(fluorosulfonyl)benzoyl]-2-aza-1,N^6-ethenoadenosine (5′-FSBaεA) reacts covalently with glutamate dehydrogenase to about 1 mol/peptide chain. As compared to native enzyme, the SBaεA–enzyme exhibits decreased sensitivity to GTP inhibition but retains its catalytic activity as well as its ability to be activated by ADP and inhibited by high concentrations of NADH. Complete protection against decreased sensitivity to GTP inhibition is provided by GTP in the presence of NADH. It is concluded that 5′-FSBaεA modifies a GTP site on glutamate dehydrogenase. The distance of 23 Å between the catalytic site labeled with ISA and a GTP site labeled with 5′-FSBaεA was measured from the quenching of salicylate donor fluorescence in the presence of the SBaεA acceptor on a doubly labeled enzyme. The average distance between the ADP and GTP sites was previously measured as 18 Å [Jacobson, M. A., & Colman, R. F. (1983) *Biochemistry 22*, 4247–4257], indicating that the regulatory sites of glutamate dehydrogenase are closer to each other than to the catalytic site.

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 (when measured in the presence of NADH), and two for NADH (one catalytic and one regulatory) (Pantaloni & Dessen, 1969; Goldin & Frieden, 1972; Pal & Colman, 1979). The relationship of the nucleotide sites has been examined kinetically, and a competition between ADP and GTP has been demonstrated (Frieden, 1963). Additional evidence has suggested that some degree of overlap or indirect interaction may exist between the various regulatory sites on the enzyme (Colman & Frieden, 1966; Cross & Fisher, 1970). A direct measurement of the distances between two sites on a protein can be achieved by fluorescence energy transfer (Förster, 1959; Stryer, 1978) employing a chromophoric pair in which the emission spectrum of the donor chromophore overlaps with the absorption spectrum of the acceptor chromophore.

The fluorescent nucleotide analogue 5′-[*p*-(fluorosulfonyl)benzoyl]-1,N^6-ethenoadenosine (5′-FSBεA) has been shown to react specifically at one of the two GTP inhibitory

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[1] Abbreviations: 5′-FSBεA, 5′-[*p*-(fluorosulfonyl)benzoyl]-1,N^6-ethenoadenosine; 5′-FSBaεA, 5′-[*p*-(fluorosulfonyl)benzoyl]-2-aza-1,N^6-ethenoadenosine; SBaεA, 5′-(*p*-sulfobenzoyl)-2-aza-1,N^6-ethenoadenosine; GTP, guanosine 5′-triphosphate; ADP, adenosine 5′-diphosphate; NADH, reduced nicotinamide adenine dinucleotide; ISA, 4-(iodoacetamido)salicylic acid; TNP-ADP, 2′,3′-*O*-(2,4,6-trinitrocyclohexadienylidene)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.

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sites on glutamate dehydrogenase (Jacobson & Colman, 1982). As compared to native glutamate dehydrogenase, the modified enzyme retains full catalytic activity and the ability to be activated by ADP and inhibited by high concentrations of NADH but exhibits a decreased affinity for GTP and a diminished but not abolished inhibition by saturating concentrations of GTP. The modified enzyme binds only 1 mol of GTP/mol of subunit in contrast to the 2 mol of GTP/mol of subunit bound by native enzyme, in the presence of NADH, demonstrating that as the result of modification by 5′-FSBεA one of the natural GTP sites is eliminated. A tyrosine has been determined to be an essential residue in the GTP binding site modified by 5′-FSBεA (Jacobson & Colman, 1983). The ADP analogue 2′,3′-*O*-(2,4,6-trinitrocyclohexadienylidene)adenosine 5′-diphosphate (TNP-ADP) was used as a fluorescent probe of the ADP site of glutamate dehydrogenase. By using the SBεA and TNP-ADP as a donor–acceptor pair, a distance of 18 Å was measured between the GTP and ADP regulatory sites (Jacobson & Colman, 1983). The utilization of other fluorescent probes would allow us to extend our estimates of the distance relationships to additional sites of glutamate dehydrogenase.

Glutamate dehydrogenase is irreversibly inactivated by 4-(iodoacetamido)salicylic acid (ISA) (Malcolm & Radda, 1970; Holbrook et al., 1973). Enzyme containing 0.99 mol of acetamidosalicylate/mol of subunit exhibited an 82% loss in initial activity, and the inclusion of the substrate α-ketoglutarate protected against the inactivation (Wallis & Holbrook, 1973). A single modified peptide was isolated, and the incorporation was attributed to modification of lysine-126 in the active site (Holbrook et al., 1973). The ISA-modified enzyme exhibits a fluorescence emission due to the covalently bound acetamidosalicylate moiety, which is spectrally compatible with the TNP-ADP absorption, allowing the distance between the catalytic and ADP sites to be evaluated in this paper.

The determination of the distance between the catalytic and GTP sites would further define the relationship between the catalytic and the two regulatory sites on glutamate dehydrogenase. The fluorescent label 5′-FSBεA previously used to modify a GTP site does not exhibit appropriate spectral overlap with ISA. The fluorescent nucleotide analogue 5′-[*p*-(fluorosulfonyl)benzoyl]-2-aza-1,N^6-ethenoadenosine (5′-FSBaεA) is structurally similar to 5′-FSBεA but possesses different spectral properties (λ_{abs} = 356 nm, λ_{emiss} = 490 nm) (Craig & Hammes, 1980) and qualifies as an acceptor for the salicylate donor fluorescence. The reaction of 5′-FSBaεA with glutamate dehydrogenase is described in this paper, and the distances between the catalytic and regulatory sites are measured by fluorescence energy transfer.

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 15 000 rpm to remove precipitated, denatured protein. The enzyme concentration was determined from 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. An M_r of 56 100 for identical peptide chains was used in the calculations (Smith et al. 1970).

4-(Iodoacetamido)salicylic acid, obtained from Nutritional Biochemicals, Inc., was recrystallized from ethanol to yield a compound with a melting point of 207–208.5 °C. Aqueous solutions were prepared by titration to pH 6.0 with sodium hydroxide. The concentration was determined spectrophotometrically with the extinction coefficients of 9.13×10^3 M^{-1} cm^{-1} at 302 nm and 1.30×10^4 M^{-1} cm^{-1} at 268 nm (Rosen et al., 1973). Radioactive 4-(iodoacetamido)salicylic acid was prepared from iodo[1-^{14}C]acetic acid (New England Nuclear Corp.) as described by Bacon et al. (1981). 5′-[*p*-(Fluorosulfonyl)benzoyl]-2-aza-1,N^6-ethenoadenosine was synthesized by the procedure of Craig & Hammes (1980); 2-aza-1,N^6-ethenoadenosine was from Molecular Probes, and *p*-(fluorosulfonyl)benzoyl chloride was from Aldrich Chemical Co. TNP-ADP and 2-aza-1,N^6-ethenoadenosine 5′-triphosphate were also 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. All other chemicals were reagent grade.

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 NADH. 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. Conditions used for testing the effects of varying concentrations of regulatory compounds, such as ADP or GTP, are indicated under Results.

Modification of Glutamate Dehydrogenase with 4-(Iodoacetamido)salicylic Acid. Glutamate dehydrogenase (2.1 mg/mL) was incubated with 0.4 mM 4-(iodo[^{14}C]acetamido)salicylic acid at 37 °C in 0.067 M sodium phosphate buffer, pH 7.5, as described previously by Holbrook et al. (1973). At various times, aliquots were removed from this reaction mixture and from a control containing no ISA and were assayed for glutamate dehydrogenase activity. After 200 min, when the enzyme had lost 80% of its original activity, the reaction was terminated by addition of β-mercaptoethanol so that the final concentration was 8 mM. The reaction mixture was allowed to sit on ice for 10 min after which the enzyme was separated from excess reagent by the column-centrifugation procedure described by Penefsky (1979), using Sephadex G-50, 80 mesh, equilibrated in Tris–0.05 M acetate, pH 8.0, containing 10 mM potassium phosphate and 100 μM EDTA. The amount of reagent incorporated was determined by measuring the radioactivity in 100-μL aliquots from the eluate obtained by the column centrifugation procedure; 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. Enzyme that had lost 80% of its initial activity contained 1.1 mol of acetamidosalicylic acid/mol of enzyme subunit.

Reaction of 5′-[p-(Fluorosulfonyl)benzoyl]-2-aza-1,N^6-ethenoadenosine with Glutamate Dehydrogenase. Glutamate dehydrogenase (1 mg/mL) was incubated with 5′-FSBaεA (1.4 mM) at 30 °C in 0.01 M sodium barbital buffer (pH 8)

containing 0.2 M KCl and 10% dimethylformamide. Dimethylformamide was required to maintain the solubility of the reagent and had no effect on the stability or activity of the enzyme as determined from a control that consisted of enzyme incubated under the same conditions but in the absence of 5′-FSBaεA. Aliquots were withdrawn at given time intervals, diluted 85-fold in Tris–0.1 M acetate (pH) at 0 °C, and assayed in the absence or presence of GTP, with 30–60 μL, respectively, in a total volume of 1.0 mL. Data presented have been normalized for any differences in protein concentration used in the assay. The extent of reaction is defined by the percent change in GTP inhibition as $(V_i - V_0)/(V_\infty - V_0) \times 100$, where V_i 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.

Incorporation of 5′-(p-Sulfobenzoyl)-2-aza-1,N⁶-ethenoadenosine by Glutamate Dehydrogenase. At a given extent of reaction, SBaεA-modified enzyme was isolated by the column-centrifugation procedure of Penefsky (1979) using Sephadex G-50, 80 mesh, equilibrated with 0.025 M potassium phosphate buffer, pH 7.1, or 0.067 M sodium phosphate buffer, pH 7.5, if the SBaεA-modified enzyme was to be used for a second modification by ISA. The protein concentration of the eluate was determined by the Bio-Rad assay as described above. The amount of incorporated reagent was determined by a fluorometric method in a thermostated cell in a Hitachi Perkin-Elmer MPF-3 spectrofluorometer at 25 °C. An aliquot of SBaεA-modified enzyme, diluted to 0.13 mg/mL in 0.3 mL, was denatured in 6 M urea. The fluorescence emission at 490 nm (λ_{excit} = 356 nm) was measured and compared to a standard curve of 5′-FSBaεA in 6 M urea. The protein concentration of SBaεA-modified enzyme was remeasured after denaturation with urea.

Identification of Amino Acid Residues Modified by 5′-[p-(Fluorosulfonyl)benzoyl]-2-aza-1,N⁶-ethenoadenosine. SBaεA-modified enzyme of determined incorporation was dialyzed against deionized water for 24 h with repeated changes. After 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 excised from the unsprayed, dry plate, extracted 5 times with water, and evaporated to dryness. A standard solution of amino acids containing known amounts of CBS-Lys and CSB-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). The aqueous dimethyl sulfoxide system (Moore, 1968) was used in detecting ninhydrin-positive components. Total amounts of protein applied to the thin-layer plate were 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).

Preparation of Glutamate Dehydrogenase Labeled with 5′-(Sulfobenzoyl)-2-aza-1,N⁶-ethenoadenosine and 4-(Iodoacetamido)salicylic Acid. SBaεA-modified enzyme was prepared and isolated in 0.067 M sodium phosphate buffer, pH 7.5, as described above. The enzyme contained 1.15 mol of SBaεA/mol of enzyme subunit and exhibited 100% change in sensitivity to GTP inhibition. This SBaεA-modified enzyme (0.85 mg/mL) was incubated with 0.4 mM 4-(iodo[^{14}C]-acetamido)salicylic acid at 37 °C in 0.067 M sodium phosphate buffer, pH 7.5. At various times, aliquots were removed from this incubation mixture and from a control of SBaεA-modified enzyme in the same buffer containing no 4-(iodoacetamido)salicylic acid. After 3 h, the SBaεA–enzyme had lost 80% of its initial activity. The activity of SBaεA-modified enzyme incubated under identical conditions but in the absence of ISA remained constant for this period of time. The reaction was terminated as before, by the addition of β-mercaptoethanol to a final concentration of 8 mM. After the reaction mixture was allowed to sit on ice for 10 min, the enzyme was separated from excess reagent by the column-centrifugation procedure using Sephadex G-50, 80 mesh, equilibrated in Tris–0.05 M acetate, pH 8.0, containing 10 mM potassium phosphate and 100 μM EDTA. The amount of [^{14}C]acetamidosalicylic acid incorporated was measured as described above. The SBaεA incorporation was also remeasured after modification by 4-(iodo[^{14}C]acetamido)salicylic acid. Different samples of doubly labeled enzyme contained 0.80 mol of SBaεA/mol of subunit and 0.60 mol of [^{14}C]acetamidosalicylic acid/mol of subunit and 0.78 mol of SBaεA/mol of subunit and 0.87 mol of [^{14}C]acetamidosalicylic acid/mol of subunit.

TNP-ADP Binding Measured by Fluorescence Titration. The binding of TNP-ADP to both native and ISA-modified enzymes was measured in Tris–0.05 M acetate buffer (pH 8.0) containing 10 mM potassium phosphate and 100 μM EDTA at 25 °C on a Perkin-Elmer 650-10S fluorescence spectrophotometer. Samples were excited at 410 nm, and emission was measured at 540 nm. The dissociation constants and number of TNP-ADP binding sites on native and ISA-modified enzymes were determined by the method described by Reynolds et al. (1978). 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 ratio of the specific fluorescence intensities of bound and free TNP-ADP. The value of Q was measured by titrating a fixed amount of TNP-ADP (3–4 μM) with increasing amounts of native or ISA-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 estimate the value of Q. The Q values for native and ISA-modified enzymes were measured to be 5.3 and 4.5, 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:

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

where $[\text{TNP-ADP}]_{\text{free}}$ 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 ISA-modified enzymes (5 μM) 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. Total concentration of [8-^{14}C]ADP used in the binding mixture was 5 μM. 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.

Fluorescence Measurements. The quantum yields of ISA-modified and SBaϵA-modified enzyme were measured on a Hitachi Perkin-Elmer MPF-44B fluorescence spectrophotometer thermostated at 25 °C and equipped with a corrected spectra accessory. A value of 0.17 for ISA-modified enzyme and 0.17 for SBaϵA-modified enzyme was obtained from the comparative method of Parker & Reese (1960) 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).

The steady-state fluorescence polarization of ISA-modified enzyme (5 μM) and SBϵA-modified enzyme (3 μM) was measured at 25 °C with an SLM 4800 spectrofluorometer (SLM Instruments, Urbana, IL). The excitation and emission wavelengths were 303 and 400 nm, respectively, for acetamidosalicylate and 310 and 405 nm for SBϵA. The polarization, P, is defined as

$$P = \frac{F_\parallel - F_\perp}{F_\parallel + F_\perp} \quad (3)$$

where $F_\parallel$ and $F_\perp$ are the fluorescence intensities when the emission polarizer is oriented in the parallel and perpendicular directions, respectively, relative to the excitation polarizer. A correction was made for the unequal transmission of horizontally and vertically polarized light by the emission monochromator at the emission wavelength of 400 and 405 nm. Corrections were also made for protein light scattering by measuring the apparent emission of 5 and 3 μM nonmodified enzyme. Values of 0.26 and 0.15 were measured for the polarization of bound acetamidosalicylate and SBϵA on glutamate dehydrogenase, respectively.

The distance between the catalytic and regulatory sites on glutamate dehydrogenase was measured in accordance with the Förster theory of energy transfer. The distance R is related to the efficiency of energy transfer E according to

$$R = R_0(E^{-1} - 1)^{1/6} \quad (4)$$

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

$$R_0 = (9.79 \times 10^3)(JK^2Q_Dn^{-4})^{1/6}\ \text{Å} \quad (5)$$

In eq 5, 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 orientation of the donor and acceptor transition dipoles, and J is the spectral overlap integral of donor fluorescence and acceptor absorption. The spectral overlap J is calculated by using the overlap integral

$$J = \frac{\sum F_D(\lambda)\epsilon_A(\lambda)\lambda^4\,\Delta\lambda}{\sum F_D(\lambda)\,\Delta\lambda} \quad (6)$$

and integrating by 5-nm intervals. In the calculation of R_0, 1.4 was used as the refractive index n. The orientation factor K^2 was assumed to be $^2/_3$, which is a calculated value for donor and acceptor dipoles rotating rapidly compared to the fluorescence lifetime of the donor (Förster, 1959). The efficiency of energy transfer was measured from the quenching of ISA fluorescence at 400 nm (303-nm excitation) in the presence of the acceptor by

$$E = 1 - Q_{DA}/Q_D \quad (7)$$

where Q_{DA} and Q_D are the quantum yields of ISA-modified enzyme in the presence and absence of an acceptor, respectively. The wavelength dependence of bound acetamidosalicylate excitation and emission is unaffected by the presence of TNP-ADP or SBaϵA; thus the ratio of fluorescence emission at 400 nm in the presence or absence of an acceptor, F_{DA}/F_D, is proportional to the ratio of the quantum yields and was used to calculate the efficiency E. The quenching of fluorescence emission at 400 nm for ISA-modified enzyme in the presence of the TNP-ADP acceptor was measured upon titration of 5 μM modified enzyme containing 1.1 mol of acetamidosalicylate/mol of subunit with 0–12.0 μM TNP-ADP at 25 °C in Tris–0.05 M acetate buffer (pH 8.0) containing 10 mM potassium phosphate and 100 μM EDTA.

The ratio of fluorescence emission at 400 nm for ISA-modified enzyme in the presence and absence of the SBaϵA acceptor was corrected for the difference in the stoichiometry of donor labeling in the singly as compared to the doubly labeled enzyme by using

$$\frac{Q_{DA}}{Q_D} = \frac{F_{DA}}{F_D}\frac{[D]_D}{[D]_{DA}} \quad (8)$$

where Q_{DA} and Q_D are the quantum yields of ISA-modified enzyme in the presence and absence of an acceptor, respectively, F_{DA} and F_D are the fluorescence emission at 400 nm (303-nm excitation) of ISA-modified enzyme in the presence and absence of an acceptor, respectively, and $[D]_D/[D]_{DA}$ is the ratio of the relative concentrations of donor in the singly labeled and doubly labeled enzymes, respectively (Fairclough & Cantor, 1978).

Results

Titration of TNP-ADP Nucleotide Binding Sites on ISA-Modified Enzyme. TNP-ADP has been demonstrated to serve as a chromophoric probe of the ADP site on native glutamate dehydrogenase (Jacobson & Colman, 1983). This ADP analogue reversibly activates native enzyme and competes with ADP both kinetically and for binding to native enzyme. The ability of TNP-ADP to bind to ISA-modified enzyme was examined by fluorometric titration as described under Experimental Procedures. Successive additions of TNP-ADP were made to the same cuvette either in the presence or absence of ISA-modified enzyme. The total fluorescence intensity was recorded after each addition. A large fluorescence enhancement is observed in the presence of 5 μM ISA-modified enzyme (Figure 1) concomitant with a blue shift of the TNP-ADP emission maximum from 560 to 540 nm. A similar

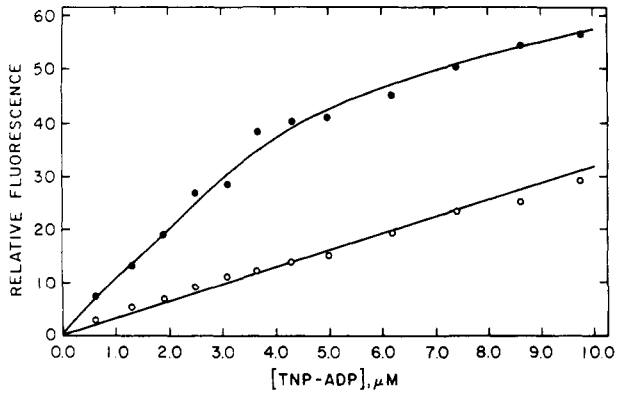

FIGURE 1: Fluorescence titration of ISA-modified enzyme with TNP-ADP. Sequential additions of TNP-ADP were made to cuvettes with (●) or without (○) 5 μM modified enzyme containing 1.1 mol of acetamidosalicylate/mol of subunit. Titrations were carried out in Tris–0.05 M acetate buffer (pH 8.0) containing 10 mM potassium phosphate and 100 μM EDTA at 25 °C.

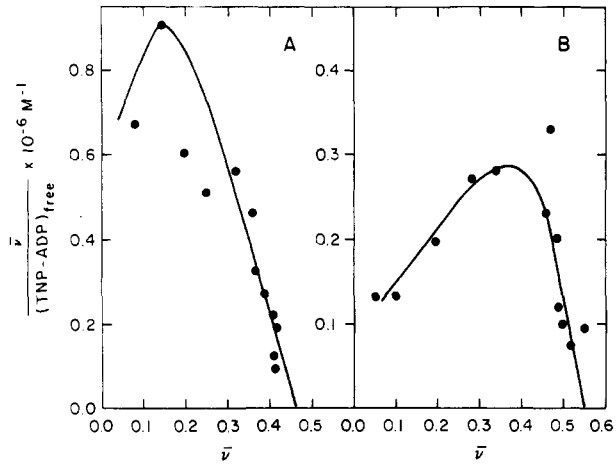

FIGURE 2: Binding of TNP-ADP to native (A) and ISA-modified (B) enzymes. Buffer conditions are as described in Figure 1. ISA-modified enzyme contained 1.1 mol of acetamidosalicylate/mol of subunit.

result has been reported for native glutamate dehydrogenase (Jacobson & Colman, 1983). The enzyme appears to be saturated by TNP-ADP since the increment of fluorescence at 540 nm with increasing TNP-ADP in the presence of enzyme approaches that of TNP-ADP in buffer alone above 6 μM (Figure 1). By using in eq 1 the ratio of TNP-ADP fluorescence measured in the presence and absence of enzyme and the Q value, determined for bound TNP-ADP, the concentration of bound TNP-ADP was calculated and used to determine $\bar{\nu}$ in eq 2.

The binding of TNP-ADP to ISA-modified enzyme is compared to that observed previously for native enzyme in Figure 2. Both enzymes exhibit nonlinear Scatchard plots, indicating an apparent cooperativity among subunits with similar affinity for and maximum number of TNP-ADP sites/mol of subunit: native enzyme binds 0.46 mol of TNP-ADP sites/mol of subunit with a K_d of 0.3 μM estimated from the limiting slope at high concentrations of TNP-ADP (Figure 2A), while ISA-modified enzyme binds 0.55 mol of TNP-ADP/mol of subunit with a K_d of 0.4 μM estimated from the limiting slope. The apparent cooperativity for binding of TNP-ADP to native enzyme has been discussed previously (Jacobson & Colman, 1983).

The amount of [8-^{14}C]ADP bound to ISA-modified enzyme is markedly decreased in the presence of saturating concentrations of TNP-ADP. The binding of [8-^{14}C]ADP was determined by an ultrafiltration technique as described under Experimental Procedures. In the absence of TNP-ADP, ISA-modified enzyme binds 0.39 mol of ADP/mol of subunit. 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 0.092 mol of ADP/mol of subunit. This result is similar to that observed for native enzyme: native enzyme binds 0.62 mol of ADP/mol of subunit in the absence of TNP-ADP as compared to 0.043 mol of ADP/mol of subunit in the presence of TNP-ADP. These results strongly indicate that TNP-ADP binds to and competes with ADP for binding to ISA-modified enzyme and can serve as a fluorescent probe of the ADP binding site on ISA-modified enzyme as has been previously shown for native glutamate dehydrogenase.

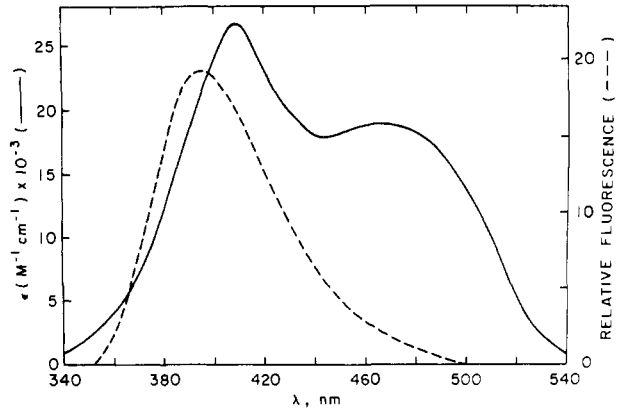

FIGURE 3: Spectral overlap of the corrected fluorescence emission of ISA-modified enzyme excited at 303 nm (– –) with the extinction coefficient (ε) of TNP-ADP (—) in Tris–0.05 M acetate buffer (pH 8.0) containing 10 mM potassium phosphate and 100 μM EDTA.

Measurement of Energy Transfer between Catalytic and ADP Sites on Glutamate Dehydrogenase. The absorption spectrum of TNP-ADP overlaps substantially with the corrected emission spectrum of ISA-modified enzyme as shown in Figure 3. The spectral overlap J was calculated from Figure 3 by using the overlap integral (eq 6) and integrating by 5-nm intervals. A spectral overlap value of 6.41×10^{-14} M^{-1} cm^3 was calculated for the acetamidosalicylic acid–TNP-ADP donor–acceptor pair. The critical transfer distance R_0 was calculated from eq 5 to be 42 Å.

The quenching of donor fluorescence from acetamidosalicylic acid covalently bound at the catalytic 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 efficiency of energy transfer was calculated by eq 7 from the quenching of the fluorescence of 5 μM ISA-modified enzyme upon titration with TNP-ADP in the range 0–12.0 μM. The observed efficiency was corrected for the fraction of maximum TNP-ADP sites occupied at a given TNP-ADP concentration as described by Fairclough & Cantor (1978). The amount of TNP-ADP bound over the concentration range used for titration was determined from the Scatchard plot (Figure 2B). Throughout the range of 2.2–12.0 μM TNP-ADP (corresponding to $\bar{\nu}$ = 0.43–0.55), the corrected efficiency of energy transfer was constant at 0.81. A value of 33 Å was calculated as the average distance between the catalytic and ADP sites on glutamate dehydrogenase by using the energy-transfer efficiency and eq 4. Titration of ISA-modified enzyme with the nonfluorescent natural activator ADP in the concentration range 0–155 μM resulted in no quenching of donor fluorescence, providing evidence that the decreased fluorescence in

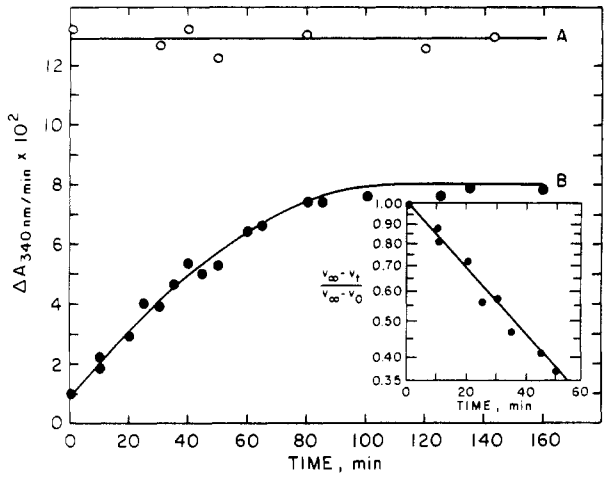

FIGURE 4: Reaction of 1.4 mM 5′-[*p*-(fluorosulfonyl)benzoyl]-2-aza-1,N^6-ethenoadenosine with glutamate dehydrogenase. Enzyme (1 mg/mL) was incubated as described under Experimental Procedures and assayed (A) in the absence and (B) in the presence of 1 μM GTP. (Inset) Determination of the pseudo-first-order rate constant from the increase in enzyme activity assayed in the presence of GTP. The increase in activity as a function of time is expressed as $(V_\infty - V_t)/(V_\infty - V_0)$, where V_t and V_0 are the velocities at a given time and at time zero and V_∞ is the constant velocity reached at complete reaction determined by successive additions of reagent.

the presence of TNP-ADP is the result of energy transfer between the acetamidosalicylic acid and TNP-ADP chromophores rather than of a conformational change in the protein induced by the acceptor.

Effect of 2-Aza-1,N^6-etheno-ATP on the Catalytic Activity of Glutamate Dehydrogenase. Glutamate dehydrogenase is reversibly inhibited by 2-aza-1,N^6-etheno-ATP (aεATP) as assayed in Tris–0.01 M acetate buffer, pH 8.0, at 25 °C with 100 μM NADH as the coenzyme. The activity of the enzyme was measured in the absence and in the presence of aεATP over a concentration range of 0.25 μM to 1.0 mM. The inhibition constant for an allosteric uncompetitive inhibitor of glutamate dehydrogenase has been shown to be numerically equal to the inhibitor concentration at which the velocity equals

$$(1/2)(V + V_I) \qquad (9)$$

where V and V_I are the maximum velocities in the absence and presence of saturating concentrations of the inhibitor, respectively (Frieden, 1963). By using eq 9, an inhibition constant for the enzyme–aεATP complex was determined as 35 μM. At saturating concentrations of aεATP, a maximum extent of inhibition of 91% was observed. The inhibitory effect of aεATP can be compared to that previously observed for 1,N^6-etheno-ATP (εATP). A maximum extent of inhibition of 91% was observed at saturating concentrations of εATP, and an inhibition constant of 22 μM was determined (Jacobson & Colman, 1982).

Reaction of 5′-[p-(Fluorosulfonyl)benzoyl]-2-aza-1,N^6-ethenoadenosine with Glutamate Dehydrogenase. The maximum velocity of glutamate dehydrogenase when assayed in the absence of allosteric modulators is unaffected by the incubation of 1.4 mM 5′-FSBaεA for 160 min (Figure 4, curve A). In contrast, a time-dependent increase was observed in the activity of the enzyme as assayed in the presence of a constant concentration of the inhibitor GTP. The rate of reaction of 5′-FSBaεA with the enzyme can be monitored by the observed time-dependent desensitization to GTP inhibition (Figure 4, curve B and inset).

To determine if the plateau observed in curve B of Figure 4 is due to the attainment of the end point for the reaction

Table I: Effect of Substrates and Modifiers on the Rate of Reaction of 5′-[*p*-(Fluorosulfonyl)benzoyl]-2-aza-1,N^6-ethenoadenosine with Glutamate Dehydrogenase[a]

additions to reaction mixture	k_{obsd} ($min^{-1} \times 10^3$)
none	29.0
20 mM α-ketoglutarate	31.0
3 mM NADH	19.0
3 mM ADP + 100 μM NADH	17.3
100 μM GTP + 100 μM NADH	no reaction

[a] Glutamate dehydrogenase (1 mg/mL) was incubated with 5′-FSBaεA (1.4 mM) at 30 °C in 0.01 M sodium barbital buffer (pH 8.0) containing 0.2 M KCl and 10% dimethylformamide. The pseudo-first-order rate constants for the reaction were determined in accordance with the inset of Figure 4 and Results.

or alternatively can be attributed to reagent decomposition, a second aliquot of reagent was added after 130 min of incubation of 1.4 mM 5′-FSBaεA with the enzyme so that the final concentration of newly added reagent was 0.7 mM. The reaction was continually monitored as described above. No change was detected in the activity of the enzyme as assayed in the presence of GTP, suggesting that the plateau represents the end point of the reaction. The value of $\Delta A_{340}/min = 0.078$ was used as the end point in the determination of a pseudo-first-order rate constant. From a semilogarithmic plot (Figure 4, inset) of the time-dependent increase in the activity of glutamate dehydrogenase as assayed in the presence of 1 μM GTP, a rate constant $k = 0.029\ min^{-1}$ was calculated for the reaction of 1.4 mM 5′-FSBaεA with the enzyme.

The effect of added substrates and regulatory effectors on the rate of reaction of glutamate dehydrogenase with 1.4 mM 5′-FSBaεA is shown in Table I. The substrate α-ketoglutarate when included in the incubation mixture at a concentration high relative to its known dissociation constant (Goldin & Frieden, 1972) does not affect the rate of reaction of 5′-FSBaεA with the enzyme. This indicates that modification by 5′-FSBaεA does not occur at the active site. At a concentration of NADH (3 mM) sufficiently high to saturate both the catalytic and regulatory NADH sites, the reaction rate is not markedly affected. Inclusion of the activator ADP together with NADH decreases the rate constant only slightly. The concentration of ADP added is much higher than its known K_D value (Frieden, 1963), and it is considered that the change in the rate constant is due to an indirect effect of ADP on modification of a site distinct from the ADP site. A combination of GTP together with reduced coenzyme NADH provides complete protection against the reaction. [Under these conditions, it has been shown that both GTP sites are occupied (Pal & Colman, 1979).] These results suggest that the reaction of 5′-FSBaεA with glutamate dehydrogenase is occurring at a GTP inhibitory site.

Incorporation of 5′-[p-(Fluorosulfonyl)benzoyl]-2-aza-1,N^6-ethenoadenosine by Glutamate Dehydrogenase and Quantum Yield of SBaεA-Modified Enzyme. The extent of covalent incorporation of 5′-FSBaεA by glutamate dehydrogenase was determined by the amount of SBaεA fluorescence as described under Experimental Procedures. Enzyme exhibiting 100% change in sensitivity to GTP inhibition contained 1.15 mol of SBaεA/mol of subunit.

The quantum yields of 0.10 for 5′-FSBaεA free in solution and 0.17 for SBaεA bound to glutamate dehydrogenase were measured in 25 mM potassium phosphate buffer, pH 7.1, by the comparative method of Parker & Reese (1970). This almost 2-fold enhancement of the 5′-FSBaεA fluorescence upon binding to the enzyme suggests that 5′-FSBaεA exists in a somewhat different conformation, possibly less stacked

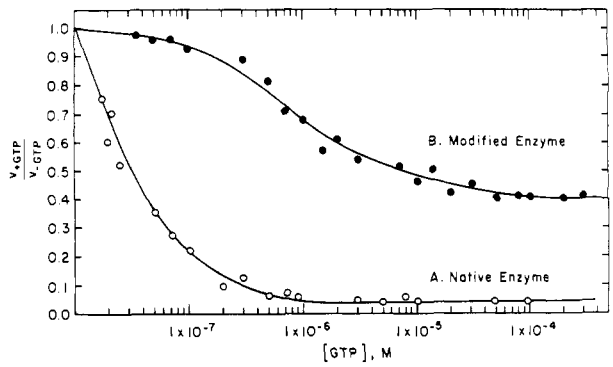

FIGURE 5: Ratio of maximum velocities in the presence and absence of GTP for native and SBaεA-modified enzymes. The velocities were measured for (A) native and (B) SBaεA-modified enzymes as described under Experimental Procedures with 100 μM NADH as the coenzyme.

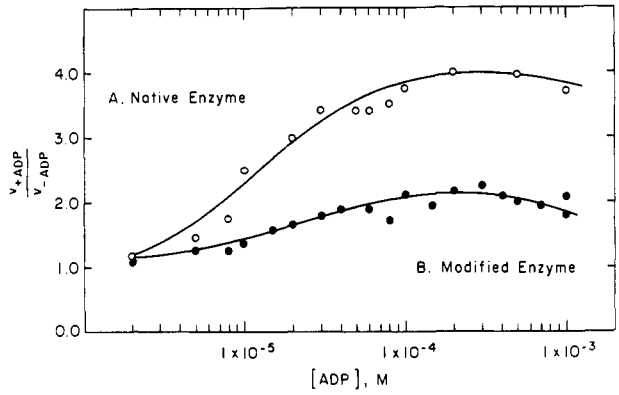

FIGURE 6: Ratio of maximum velocities in the presence and absence of ADP as a function of ADP concentration for native and SBaεA-modified enzymes. The velocities were measured for (A) native and (B) SBaεA-modified enzyme as described under Experimental Procedures with 100 μM NADPH as the coenzyme.

when enzyme bound than it does free in solution.

Kinetic Properties of SBaεA-Modified Enzyme. In the attempt to characterize the site of 5′-FSBaεA modification, the catalytic and regulatory properties of SBaεA-modified enzyme containing 1.15 mol of SBaεA/mol of subunit were compared to those observed for native enzyme. To determine if there is any alteration in the affinity of SBaεA-modified enzyme for GTP, the velocities of native and SBaεA-miodified enzymes as a function of GTP concentration were measured and compared as shown in Figure 5. The dissociation constant for the enzyme–GTP complex is determined from the relationship given in eq 9. Native enzyme (Figure 5, curve A) is inhibited by GTP to a maximum extent of 96% at saturating concentrations of GTP with K_{GTP} of 0.025 μM. In comparison, SBaεA-modified enzyme exhibits a decreased sensitivity to GTP inhibition: K_{GTP} of 0.9 μM with maximum extent of inhibition at saturating concentration of GTP of 60%. These results are consistent with reaction of 5′-FSBaεA at a GTP inhibitory site.

The velocities of both native and SBaεA-modified enzymes were measured as a function of ADP concentration and are compared in Figure 6. The dissociation constant for the enzyme–ADP complex has been shown to be numerically equal to the concentration of ADP at which the velocity equals

$$(1/2)(V + V_A) \qquad (10)$$

where V and V_A are the maximum velocities in the absence and presence of saturating concentrations of the activator, respectively (Frieden, 1963). As indicated in Figure 6, native and SBaεA-modified enzymes show a similar affinity for ADP:

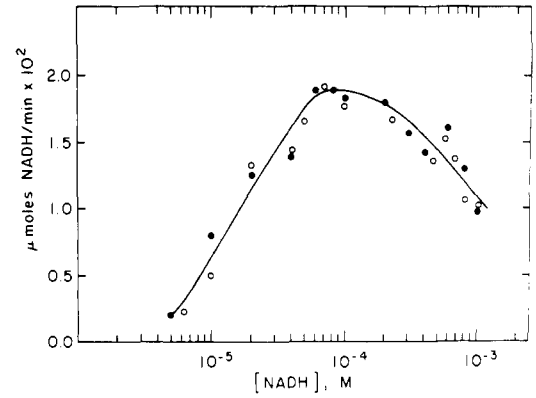

FIGURE 7: Initial velocity as a function of NADH concentration for native (○) and SBaεA-modified enzyme (●). At NADH concentrations up to 2×10^{-4} M, velocity measurements were made at 340 nm as described under Experimental Procedures with the value $\epsilon_{340} = 6.22 \times 10^3$ M^{-1} cm^{-1} for NADH. At higher NADH concentrations, measurements were made at 375 nm with the value of $\epsilon_{275} = 1.85 \times 10^3$ M^{-1} cm^{-1} for NADH being used in calculating the rates in units of micromoles of NADH per minute.

K_{ADP} for native enzyme is 15 μM and for SBaεA-modified enzyme is 16 μM. However, the maximum extent of activation is decreased significantly to 2.1-fold in SBaεA-modified enzyme as compared with a 4-fold activation observed for native enzyme. It is possible that the decrease in the extent of ADP activation observed for SBaεA-modified enzyme is the result of an indirect effect produced by modification at a separate GTP site.

Glutamate dehydrogenase is inhibited by high concentrations of NADH, which bind to a second site distinct from the catalytic site (Goldin & Frieden, 1972). Figure 7 shows that SBaεA-modified and native enzymes have a similar affinity for NADH and that both are inhibited by high concentrations of the reduced coenzyme. Although a small decrease in the rate of reaction of 5′-FSBaεA with glutamate dehydrogenase was observed in the presence of high concentrations of NADH (Table I), these results indicate that 5′-FSBaεA does not react at the NADH inhibitory site.

Identification of Amino Acid Residues of Glutamate Dehydrogenase Modified by 5′-[p-(Fluorosulfonyl)benzoyl]-2-aza-1,N⁶-ethenoadenosine. Upon acid hydrolysis of SBaεA-modified glutamate dehydrogenase, the ester linkage between the benzoyl and nucleoside moieties of SBaεA 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 1.15 mol of SBaεA/mol of subunit and exhibiting 100% change in sensitivity to GTP inhibition was analyzed for content of CBS residues. CBS-Tyr, 0.63 mol, and CBS-Lys, 0.52 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′-FSBaεA. The formation of CBS-Tyr and CBS-Lys was determined as a function of time of incubation with 5′-FSBaεA and change in GTP inhibition (Table II). The ratio of CBS-Tyr to the total amount of CBS residues (CBS-Lys + CBS-Tyr) remains relatively constant at 0.5 throughout the course of the reaction. These results suggest that tyrosine and lysine are present in a GTP inhibitory site and that 5′-FSBaεA, once bound to the site, may react with either but not both.

The amount of CBS-Tyr and CBS-Lys was also quantified in enzyme incubated with 5′-FSBaεA in the presence of 100

Table II: Quantification of CBS-Tyr and CBS-Lys in SBaεA-Modified Glutamate Dehydrogenase

% change in GTP inhibition	total incorporation (mol of SBaεA/mol of subunit)	mol of CBS-Tyr/ mol of subunit[a]	mol of CBS-Lys/ mol of subunit[a]
25	0.47	0.23	0.24
50	0.68	0.30	0.38
70	0.90	0.45	0.45
100	1.15	0.63	0.52

[a] Moles of CBS-Tyr or CBS-Lys per mole of subunit are calculated by multiplying the fraction of derivatized amino acid by the total incorporation of SBaεA per mole of subunit as determined by fluorescence.

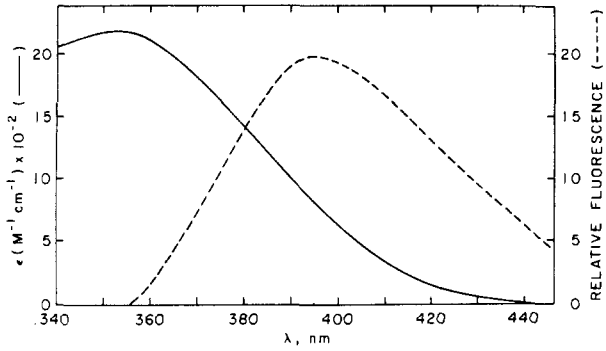

FIGURE 8: Spectral overlap of the corrected fluorescence emission of ISA-modified enzyme excited at 303 nm (– –) with the extinction coefficient (ε) of SBaεA-modified enzyme (—) in Tris–0.05 M acetate buffer (pH 8.0) containing 10 mM potassium phosphate and 100 μM EDTA.

μM GTP and 100 μM NADH, ligands that protect completely against the change in GTP inhibition. Under these conditions, the total incorporation measured was 0.38 mol of SBaεA/mol of subunit, which is decreased in comparison to enzyme modified under the same conditions except for the absence of protecting ligands. The sum of CBS-Tyr (0.17 mol/mol of subunit) and CBS-Lys (0.21 mol/mol of subunit) was found to account for the total incorporation prior to acid hydrolysis. The ratio of CBS-Tyr to the total amount of CBS residues for enzyme modified under protected conditions is also about 0.5.

Measurement of Energy Transfer between the Catalytic Site and a GTP Site on Glutamate Dehydrogenase. The absorption spectrum of SBaεA-modified enzyme overlaps the corrected fluorescence emission spectrum of ISA-modified enzyme as shown in Figure 8. The spectral overlap J of 1.7×10^{-15} M^{-1} cm^3 was calculated from eq 6. From eq 5, an R_0 value of 23 Å was determined.

The distance between the catalytic site and a GTP site was measured after preparation of a doubly labeled enzyme containing acetamidosalicylic acid and SBaεA. Doubly labeled enzyme was prepared as described under Experimental Procedures. The fluorescence at 400 nm (excitation at 303 nm) of 5 μM enzyme containing SBaεA at a GTP site and acetamidosalicylic acid at the catalytic site was measured and compared to the fluorescence of ISA-modified enzyme under the same conditions. The fluorescence was normalized for any differences in the stoichiometry of donor labeling in the singly as compared to the doubly labeled enzymes. In the presence of the SBaεA acceptor, the acetamidosalicylic acid fluorescence is decreased. An efficiency of energy transfer of 0.50 was calculated from eq 7, and an average distance between the two sites was determined from eq 4 to be 23 Å. The same efficiency of energy transfer was measured for the different preparations of doubly labeled enzyme, and therefore, it is likely that the salicylate donors are quenched by the SBaεA acceptors in proximity to them.

Discussion

The reaction of 5′-FSBaεA with glutamate dehydrogenase is very similar to that previously shown for the affinity label 5′-FSBεA (Jacobson & Colman, 1982). For both analogues, evidence has been provided implicating reaction at a GTP inhibitory site on glutamate dehydrogenase. Incubation of 5′-FSBaεA or 5′-FSBεA with glutamate dehydrogenase results in a time-dependent desensitization to GTP inhibition. The reaction of 5′-FSBaεA with glutamate dehydrogenase exhibits the same sensitivity to added substrates and modifiers as previously observed for 5′-FSBεA, with a combination of GTP and NADH providing complete protection against the reaction. The characteristics of the SBaεA-modified enzyme are also similar to those observed for SBεA-modified enzyme. Both modified enzymes have an altered affinity for and decreased extent of maximum inhibition by GTP, a decreased maximum extent of activation with no change in affinity for ADP, and a normal ability to be inhibited by high concentrations of NADH. The K_{GTP} determined for SBaεA-modified enzyme is slightly larger (0.9 μM) than that previously measured for SBεA-modified enzyme (0.37 μM). The maximal extent of inhibition at saturating concentrations of GTP is also lower for SBaεA-modified enzyme (60%) than SBεA-modified enzyme (70%). From the comparison of the kinetic properties of SBaεA-modified enzyme to native and SBεA-modified enzymes, it is concluded that 5′-FSBaεA modifies a GTP inhibitory site on glutamate dehydrogenase, in a manner similar to that observed for 5′-FSBεA.

The extent of covalent incorporation is also similar for the reactions of 5′-FSBaεA and 5′-FSBεA with glutamate dehydrogenase with 1.15 mol of SBaεA/mol of subunit and 1.28 mol of SBεA/mol of subunit incorporated at maximal change in GTP inhibition, respectively. Under conditions that completely protect against the reactions of 5′-FSBaεA and 5′-FSBεA (100 μM GTP and 100 μM NADH), the enzyme incorporates 0.38 mol of SBaεA/mol of subunit and 0.35 mol of SBεA/mol of subunit, respectively. It has previously been shown that the residues labeled by 5′-FSBεA in the presence of protecting ligands are not the same residues labeled in the absence of protectants (Jacobson & Colman, 1983). It was concluded that the moles of reagent incorporated upon modification in the presence of GTP and NADH were not included in the total incorporation resulting from reaction in the absence of ligands, which is correlated with the maximal change in GTP inhibition. On the basis of the similarities of the reactions of 5′-FSBaεA and 5′-FSBεA with glutamate dehydrogenase and the similar extent of enzyme labeling in the absence and presence of protecting ligands, it is likely that the SBaεA incorporated in the presence of protecting ligands also does not contribute to the total SBaεA incorporation in the absence of ligands.

Although the reactions of 5′-FSBaεA and 5′-FSBεA with glutamate dehydrogenase exhibit similar characteristics and clearly modify a GTP site on the enzyme, some differences exist between the reaction of these analogues. The stoichiometry of the reactions is similar; however, the distribution of tyrosine and lysine modified in the SBaεA- and SBεA-modified enzyme differs significantly. The total SBεA incorporation at maximal change in GTP inhibition has been shown to be composed of 0.95 mol of CBS-Tyr/mol of subunit and 0.33 mol of CBS-Lys/mol of subunit (Jacobson & Colman, 1983). As a function of time of incubation of 5′-FSBεA

with the enzyme, the formation of CBS-Tyr was directly proportional to the percent change in GTP inhibition. 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. In the SBaεA-modified enzyme, a tyrosine and lysine residue are almost equally labeled at maximal change in GTP inhibition and as a function of time of incubation with 5′-FSBaεA. These results suggest that both tyrosine and lysine residues are present in the GTP binding site and the reagent, once bound, can react with either but not both, thus accounting for the equal distribution of SBaεA moieties on tyrosine and lysine. A similar result has been observed for the modification of glutamate dehydrogenase with 5′-[(*p*-fluorosulfonyl)benzoyl]adenosine (Saradambal et al., 1981). The adenosine analogue reacts at the NADH inhibitory site, and the total incorporation was demonstrated to be equally divided between lysine and tyrosine. It is not surprising that the reactions of 5′-FSBεA and 5′-FSBaεA with glutamate dehydrogenase are similar since these compounds differ only by a nitrogen substituted for a carbon atom at the C-2 position of the purine ring of 5′-FSBaεA. It can be considered, however, that, even though both analogues react at the same site on the enzyme, differences may exist in the mode of binding for 5′-FSBaεA due to the nitrogen substitution. Additional evidence supporting a distinction in the mode of 5′-FSBaεA and 5′-FSBεA binding to glutamate dehydrogenase is provided by the quantum yields of bound SBaεA and SBεA. The observed 2-fold enhancement of the quantum yield of 5′-FSBaεA when bound to glutamate dehydrogenase (as compared to the compound free in solution) strongly suggests that on the enzyme this analogue exists in a different conformation than does 5′-FSBεA, for which the same quantum yield of 0.01 was measured when enzyme bound or free in solution (Jacobson & Colman, 1984); SBaεA appears to exist in a less stacked conformation than does SBεA when bound to glutamate dehydrogenase. The distinction between the conformations of these bound analogues detected by fluorescence suggests that 5′-FSBaεA and 5′-FSBεA bind somewhat differently to glutamate dehydrogenase, which may be responsible for the difference in the proportion of tyrosine and lysine modified by these analogues.

The introduction of fluorescent probes at specific sites on glutamate dehydrogenase has led to an estimation of the relative distances between sites on the enzyme by resonance energy transfer. The distance between a GTP inhibitory site covalently labeled with SBεA and the ADP activatory site occupied reversibly by TNP-ADP has been measured as 18 Å (Jacobson & Colman, 1983). The use of additional compatible donor–acceptor pairs has now allowed an estimation of the distance relationship between either the GTP or ADP regulatory site and the catalytic site.

Glutamate dehydrogenase has been shown previously to be irreversibly inactivated by the reaction of 4-(iodoacetamido)salicylic acid (Holbrook et al., 1973). Enzyme exhibiting an 82% loss in the initial activity incorporated about 1 mol of acetamidosalicylate/mol of subunit. A single peptide containing modified lysine-126 was observed and isolated, and the inactivation was attributed to the modification of this residue at the active site (Holbrook et al., 1973). The ISA-modified enzyme exhibits a fluorescence emission maximum at 400 nm upon excitation at 303 nm due to the covalently bound salicylate moiety (Malcolm & Radda, 1970). The emission spectrum of ISA-modified enzyme overlaps substantially with the absorption spectrum of TNP-ADP and constitutes a suitable donor–acceptor pair for energy-transfer measurements. TNP-ADP has been reported to activate reversibly and to compete with ADP for binding to native glutamate dehydrogenase and has been established as a chromophoric probe of the ADP site (Jacobson & Colman, 1983). The maximum number of TNP-ADP sites (about 0.5 mol of TNP-ADP/mol of subunit) on ISA-modified enzyme and the affinity for binding (0.4 μM) are comparable to those observed for native enzyme. TNP-ADP also competes with ADP for binding to ISA-modified enzyme and is a probe of the ADP site.

The distance between the catalytic and ADP sites on glutamate dehydrogenase was determined as 33 Å from the quenching of salicylate donor fluorescence upon addition of TNP-ADP. This value represents the distance between the salicylate moiety at the catalytic site and the trinitrophenyl group linked to the ribose of TNP-ADP at the ADP site. The difference in donor–acceptor stoichiometry (1 mol of salicylate/0.5 mol of TNP-ADP) must be considered in the interpretation of the 33-Å distance. The distribution of donor and acceptor molecules on the hexameric glutamate dehydrogenase is one salicylate donor bound to all six subunits, with TNP-ADP acceptor bound to only three of these. The decrease in donor fluorescence produced by the acceptor molecules is represented by the efficiency of energy transfer. If it is assumed that two donor molecules are equidistant from each acceptor, the efficiency of energy transfer is the same as the observed efficiency calculated from the quenching of donor fluorescence. 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). The calculated efficiency of energy transfer between the salicylate donors and TNP-ADP acceptors is greater than 0.5 and therefore must reflect some contributions by all the donors; it is possible, however, that the contributions of the donors are not equal. The greater probability of a donor being quenched by an acceptor located on the same subunit suggests that this is the major contribution to energy transfer. Therefore, the 33-Å value is probably an upper limit of the distance between the catalytic and ADP sites within one subunit.

The fluorescence emission of ISA-modified enzyme also overlaps with the absorption of SBaεA-modified enzyme, and the two chromophores constitute a donor–acceptor pair for energy-transfer measurements. To evaluate the distance between the catalytic and GTP sites, a doubly labeled enzyme was prepared with SBaεA at the GTP site and salicylate at the catalytic site. In the presence of the SBaεA acceptor, the fluorescence of the ISA-modified enzyme was quenched, and a distance of 23 Å between the sites was calculated. This value represents the distance between the salicylate moiety at the catalytic site and the purine ring of 5′-SBaεA at the GTP site. The stoichiometry of donors to acceptors for the distance measurement was about 1:1; however, the 1 mol of donor per subunit is divided almost equally between a tyrosine and lysine residue. It appears likely that both residues are located within the GTP binding site, probably within close proximity of each other, since they are equally susceptible to modification by 5′-FSBaεA. Additionally, it is probable that all the salicylate donors are equally quenched by the SBaεA acceptors in the GTP site and that the 23-Å value is a reasonable estimate of the distance between the GTP inhibitory and catalytic sites.

The uncertainty in the orientation factor K^2 used in calculating the critical transfer distance R_0 limits the accuracy of a distance measured by resonance energy transfer. 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). It is possible to determine the upper and lower limits for K^2 from the fluorescence polarization of the energy donor from which a range of R_0 values can be calculated (Dale et al. 1979). The polarization of enzyme-bound acetamidosalicylate was determined to be 0.26 and that of enzyme-bound SBϵA to be 0.15. Since 5′-FSBaϵA and 5′-FSBϵA are structurally related and react at the same site of glutamate dehydrogenase, it is likely that the polarizations of these two enzyme-bound sulfonylbenzoyl nucleoside derivatives are similar. These results indicate that although the chromophores are covalently bound, they still exhibit some rotational mobility. For comparison, a polarization of 0.50 would be expected for a chromophore that is rigidly bound and has no rotational freedom and a value of 0 would be expected for a chromophore that can assume all orientations during the lifetime of the donor excited state. The value of $^2/_3$ for K^2 was utilized in the calculation of the distance between sites on glutamate dehydrogenase. Haas et al. (1978) have evaluated the error in the determination of distances by energy transfer for chromophores that have a fixed but unknown orientation. In general, if the fluorescence polarization of the donor or acceptor is less than 0.3, the uncertainty in the value of K^2 may produce an error in the distances measured by energy transfer, which is not likely to be greater than ±10%. The measured values for the polarization of enzyme-bound acetamidosalicylate and SBϵA are within the limits established for polarization values of chromophores for which the assumption of $K^2 = {}^2/_3$ is reasonable. Therefore, the assumption of the value of K^2 probably produces an uncertainty not greater than ±10% in the distances measured by energy transfer. A similar conclusion was reached earlier by Matsumoto & Hammes (1975).

The distance of 18 Å previously determined between the GTP and ADP regulatory sites suggested that these sites are nonidentical and relatively close to one another (Jacobson & Colman, 1983). The estimation of the distance between the regulatory and the catalytic sites presented here further defines the proximity of these nucleotide binding sites on glutamate dehydrogenase. The distance of 33 Å between the ADP and catalytic sites and 23 Å between the GTP and catalytic sites suggests that the regulatory sites are closer to each other as compared with their distances from the catalytic site. This result is in agreement with models proposed for the relationship between the various sites on glutamate dehydrogenase derived from kinetic evidence (Frieden, 1963; Colman & Frieden, 1966; Goldin & Frieden, 1971) and the binding of allosteric effectors and substrates as monitored by ultraviolet difference spectroscopy (Cross & Fisher, 1970). The dimensions of an individual subunit of the hexameric enzyme have been estimated by low-angle X-ray analysis as 66 Å for the major axis and 43 Å for the minor axis (Sund et al., 1969; Eisenberg, 1970). It is therefore possible that the distances measured between the catalytic and regulatory sites of glutamate dehydrogenase reside within a single subunit. The distances measured between these sites further substantiate the allosteric model of regulation proposed for glutamate dehydrogenase whereby nucleotide effectors are bound to sites other than the catalytic site. A mechanism by which these nucleotides modulate the activity of the enzyme by specific conformational changes or by steric exclusion must be further evaluated.

Registry No. ISA, 4323-00-6; TNP-ADP, 77450-67-0; 5′-FSBaϵA, 72561-49-0; ADP, 58-64-0; GTP, 86-01-1; EC 1.4.1.3, 9029-12-3; α-ketoglutaric acid, 328-50-7.

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Interference of Active Site Specific Reagents in Plasminogen–Streptokinase Active Site Formation†

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ABSTRACT: We have recently observed slow, non-Michaelis–Menten kinetics of activation of native cat plasminogen by catalytic concentrations of streptokinase. In order to understand the reasons for this phenomenon, we undertook to study the formation of the plasminogen–streptokinase activator complex under the same plasminogen activation conditions. The results obtained in this study show that the potential active site in both cat and human plasminogen is capable of binding strongly the specific substrates (S) *p*-nitrophenyl *p*-guanidinobenzoate (NPGB) and H-D-valyl-L-leucyl-L-lysyl-*p*-nitroanilide, though the active site is incapable of hydrolyzing these substrates. Binding studies support these and the following conclusions. Streptokinase binds to this zymogen–substrate complex to create the ternary plasminogen–S–streptokinase complex, which then slowly converts to an acylated plasminogen–streptokinase form. This acylation reaction is 550 times slower than acylation of the preformed plasminogen–streptokinase complex by NPGB. The same reaction also occurs with human plasminogen, though the acylation reaction is 10 times faster than when the cat zymogen is used. NPGB binds specifically to plasminogen but not to streptokinase. These studies proved that inhibition of cat plasminogen activation by streptokinase occurs at the level of activator complex formation. We conclude from our studies that streptokinase binding to both cat and human plasminogen occurs at the potential active site of the zymogen. Consequently, it is probable that plasminogen activation in vivo is inhibited by binding of active site specific inhibitors to plasminogen.

The plasma zymogen plasminogen is activated to the corresponding serine protease enzyme form, plasmin, by proteolytic cleavage of its Arg_{560}–Val peptide bond. This reaction is catalyzed efficiently by urokinase or by a noncovalent binary complex formed by plasminogen with the bacterial protein streptokinase. Streptokinase itself has never been shown to possess any enzymatic activity. The equimolar plasminogen–streptokinase complex has a serine protease active site with a high specificity for activating plasminogen to plasmin via cleavage of the Arg_{560}–Val scissile bond. This active site, formed upon noncovalent complexing of the inactive zymogen with streptokinase, is in all respects a fully functional active site, and it can be titrated with *p*-nitrophenyl *p*-guanidinobenzoate (NPGB)[1] (McClintock & Bell, 1971; Reddy & Markus, 1972; Schick & Castellino, 1974). The active site generated in the complex resides in the plasminogen moiety (Morris et al., 1981), since the complex, once formed, can be dissociated and the plasminogen moiety is still active after separation from streptokinase (Summaria et al., 1982).

Both trypsinogen and chymotrypsinogen are able to bind and to hydrolyze ester substrates, including NPGB, by a mechanism identical with that of the corresponding enzyme. These reactions proceed orders of magnitude more slowly with the zymogen than with the enzyme (Gertler et al., 1974; Kerr et al., 1975; Robinson et al., 1975). Bode & Huber (1976) and Bode et al. (1978) showed that upon strong ligand binding by trypsinogen, there is a transition of the tertiary structure of the enzyme to a trypsin-like state. This transition could be induced in trypsinogen by specific short peptides which resemble the amino terminus of the enzyme or by strong active site inhibitors like pancreatic trypsin inhibitor.

We have previously reported on some properties of a plasminogen zymogen species with an active site which was isolated from Cohn fraction $III_{2,3}$ (Wohl et al., 1977). More recently, we reported on a plasminogen zymogen species with an active site, which was isolated after its active site was formed by complex formation with streptokinase (Summaria et al., 1982). These last reports verified that it is possible to have a functional active and binding site in the plasminogen zymogen molecule without hydrolysis of the Arg_{560}–Val peptide bond but gave no indication that such a binding site may be of importance in the regulation of plasminogen activation.

Previous studies also showed that the plasminogen–streptokinase complex is a noncovalent stoichiometric complex which is formed without any proteolytic cleavage (Robbins & Summaria, 1970). They did not show, however, how the active site is generated, or where the binding of streptokinase occurs. There are no detailed studies of the process of formation of the active plasminogen–streptokinase complex.

Our observation that cat plasminogen–streptokinase complex titration with NPGB is not instantaneous when streptokinase is added last to the reaction mixture provided us with a way

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[1] Abbreviations: IU, international unit(s), established by the World Health Organization for streptokinase; NPGB, *p*-nitrophenyl *p*-guanidinobenzoate; H-D-Val-Leu-Lys-pNA, H-D-valyl-L-leucyl-L-lysyl-*p*-nitroanilide; Plg, plasminogen; SK, streptokinase.

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