Rossotti, F. J. C., & Rossotti, H. (1961) in *The Determination of Stability Constant*, p 58, McGraw-Hill, New York.
Schafer, R., & Zillig, W. (1973) *Eur. J. Biochem. 33*, 201.
Scrutton, M. C., Wu, C.-W., & Goldthwait, D. A. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 2497.
Siebenlist, U. (1979) *Nucleic Acids Res. 6*, 1895.
Sigman, D. S., Graham, D. R., D'Aurora, V., & Stern, A. M. (1979) *J. Biol. Chem. 254*, 12269.
Sillen, L. G., & Martell, A. E. (1964) in *Stability Constants of Metal Ion Complexes*, Special Publication No. 17, The Chemical Society, London.
Speckhard, D. C., Wu, F. Y.-H., & Wu, C.-W. (1977) *Biochemistry 16*, 5228.
Spector, T. (1978) *Anal. Biochem. 86*, 142.
Wu, C.-W., Wu, F. Y.-H., & Speckhard, D. C. (1977) *Biochemistry 16*, 5449.
Wu, F. Y.-H., & Wu, C.-W. (1973) *Biochemistry 12*, 4343.
Wu, F. Y.-H., & Wu, C.-W. (1981) *Adv. Inorg. Biochem. 3*, 143.
Wu, F. Y.-H., & Wu, C.-W. (1983) *Met. Ions Biol. Syst. 15*, 157.
Yarbrough, L. R., & Hurwitz, J. (1974) *J. Biol. Chem. 249*, 5394.
Zillig, W., Zechel, K., Rabussay, P., Schachner, M., Sethi, V. S., Palm, P., Heil, H., & Seifter, W. (1970) *Cold Spring Harbor Symp. Quant. Biol. 35*, 47.

Isolation and Identification of a Tyrosyl Peptide Labeled by 5′-[*p*-(Fluorosulfonyl)benzoyl]-1,N^6-ethenoadenosine at a GTP Site of Glutamate Dehydrogenase†

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ABSTRACT: The fluorescent nucleotide analogue 5′-[*p*-(fluorosulfonyl)benzoyl]-1,N^6-ethenoadenosine (5′-FSBεA) was shown previously to react at a GTP inhibitory site on bovine liver glutamate dehydrogenase. The incorporation was limited to 1.28 mol of reagent/mol of subunit and was attributed to 0.95 mol of modified tyrosine/mol of subunit and 0.33 mol of modified lysine/mol of subunit, quantitatively accounting for the total incorporation prior to acid hydrolysis [Jacobson, M. A., & Colman, R. F. (1983) *Biochemistry 22*, 4247–4257]. The specific tyrosyl peptide modified by 5′-FSBεA has been isolated from a tryptic and chymotryptic digest of modified enzyme by gel filtration and reverse-phase high-performance liquid chromatography and characterized by amino acid and amino-terminal analysis. A unique residue, tyrosine-262, was identified as an essential amino acid within the GTP binding site. The stacked conformation of the fluorescent analogue when enzyme bound suggests that tyrosine-262 may be located in the region of the GTP site which binds the purine ring.

The activity of the allosteric enzyme bovine liver glutamate dehydrogenase [L-glutamate:NAD(P)$^+$ oxidoreductase (deaminating), EC 1.4.1.3] is modulated by GTP, which inhibits, ADP, which activates, and NADH, which inhibits at high concentrations by binding at a site distinct from the catalytic site (Goldin & Frieden, 1972). The enzyme is composed of six identical subunits with several nucleotide sites per subunit, including one for ADP, two for GTP, and two for NADH (one catalytic and one regulatory) (Goldin & Frieden, 1972; Pantaloni & Dessen, 1969; Pal & Colman, 1979). In the attempt to identify essential amino acid residues in the regulatory sites of glutamate dehydrogenase, the reactions of several purine nucleoside affinity labels with the enzyme have been studied (Colman, 1983). The fluorescent nucleotide analogue 5′-[*p*-(fluorosulfonyl)benzoyl]-1,N^6-ethenoadenosine (5′-FSBεA)[1] has been shown to react specifically at one of the GTP sites on glutamate dehydrogenase (Jacobson & Colman, 1982) and has been utilized as a fluorescent probe in the estimation of distances by fluorescence energy transfer between this site and the ADP activator site (Jacobson & Colman, 1983). As compared to native glutamate dehydrogenase, modified enzyme retains full catalytic activity and normal ability to be inhibited by high concentrations of NADH but exhibits a decreased affinity for and diminished maximum inhibition by saturating concentrations of GTP and a decreased maximum extent of activation with no change in affinity for ADP. Enzyme with 1.28 mol of 5′-(*p*-sulfonylbenzoyl)-1,N_6-ethenoadenosine incorporated/mol of subunit and exhibiting maximum change in sensitivity to GTP inhibition has been shown 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 (Jacobson & Colman, 1983). 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. Thus, it was concluded that the tyrosine is an essential residue in the GTP binding site of glutamate dehydrogenase, while the

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[1] Abbreviations: 5′-FSBεA, 5′-[*p*-(fluorosulfonyl)benzoyl]-1,N^6-ethenoadenosine; 5′-SBεA, 5′-(*p*-sulfonylbenzoyl)-1,N^6-ethenoadenosine; CBS-Tyr, *O*-[(4-carboxyphenyl)sulfonyl]tyrosine; CBS-Lys, N^ϵ-[(4-carboxyphenyl)sulfonyl]lysine; HPLC, high-performance liquid chromatography; TPCK, tosylphenylalanine chloromethyl ketone.

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lysine modified is not involved in the inhibitory action of GTP. The present study describes the isolation and characterization of the essential tyrosyl peptide of glutamate dehydrogenase modified by 5′-FSBϵA.

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, 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 by using the value $E^{1\%}_{279}$ = 9.7 (Olson & Anfinsen, 1952); the ratio A_{280}/A_{260} was 1.9. A molecular weight of 56 100 for identical peptide chains was used in the calculations (Smith et al., 1970).

p-Amino[*carboxy*-^{14}C]benzoic acid was purchased from *ICN*. Dithiothreitol, iodoacetic acid, and chymotrypsin were obtained from Sigma Chemical Co. TPCK–trypsin was purchased from Worthington. Ultrapure urea was obtained from Schwarz/Mann, Inc. Trifluoroacetic acid and dansyl chloride were purchased from Pierce. HPLC-grade acetonitrile was obtained from Burdick & Jackson. Nonradioactive 5′-FSBϵA was synthesized as previously described (Jacobson & Colman, 1982). 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.

Synthesis of [p-(Fluorosulfonyl)[^{14}C]benzoyl]-1,N^6-ethenoadenosine. The reagent 5′-FSBϵA was synthesized with the radioactive label in the carboxyl group of the benzoyl moiety starting with *p*-amino[*carboxyl*-^{14}C]benzoic acid. *p*-(Fluorosulfonyl)[^{14}C]benzoyl chloride was synthesized according to the procedure of Esch & Allison (1978) starting with 0.5 mCi of *p*-amino[^{14}C]benzoic acid diluted to 1.0 mmol with cold *p*-aminobenzoic acid. [^{14}C]-5′-FSBϵA was synthesized by the procedure described previously for 5′-FSBϵA (Jacobson & Colman, 1982) with the following modification: 100 mg (0.31 mmol) of ethenoadenosine was dissolved in 0.7 mL of hexamethylphosphoramide with warming to 50 °C. After being cooled to room temperature, this solution was added to the synthesized *p*-(fluorofulsonyl)[^{14}C]benzoyl chloride (0.43 mmol) and incubated for 18 h at room temperature with stirring. A single addition of *p*-(fluorosulfonyl)benzoyl chloride was made to the solution of ethenoadenosine in contrast to the procedure followed for nonradioactive 5′-FSBϵA (Jacobson & Colman, 1982). The product, [^{14}C]-5′-FSBϵA, was detected by thin-layer chromatography on 200-μm silica gel plates (EM Reagents) using methyl ethyl ketone–acetone–H_2O (65:20:15) as the solvent. The reaction mixture was extracted 3 times with 3 mL of petroleum ether. The product was purified on 1500-μm silica gel plates (Analtech, Inc.) as described previously (Jacobson & Colman, 1982). The specific activity of the final product, [^{14}C]-5′-FSBϵA, was 1.02 × 10^{12} cpm/mol.

Preparation of 5′-SBϵA Modified Enzyme. Glutamate dehydrogenase (1 mg/mL) was incubated with 1.4 mM 5′-FSBϵA or [^{14}C]-5′-FSBϵA at 30 °C in 0.01 M sodium barbital buffer (pH 8.0) containing 0.2 M KCl and dimethylformamide as described previously (Jacobson & Colman, 1982). After 120 min, a second aliquot of 5′-FSBϵA was added so that the final concentration of new reagent was 0.7 mM. The final concentration of dimethylformamide was 10%. One hour after the second addition of reagent, the modified enzyme was isolated by the column centrifugation technique (Penefsky, 1979) using Sephadex G-50-80 equilibrated with 0.025 M potassium phosphate buffer, pH 7.1. The amount of covalent incorporation was determined by either a fluorometric or radiochemical method as described previously (Jacobson & Colman, 1983).

Carboxymethylation of 5′-SBϵA Modified Enzyme. Modified enzyme (10–32 mg) was dialyzed overnight at 4 °C against 50 mM NH_4HCO_3, pH 8.0. The sulfhydryl residues of the modified enzyme were carboxymethylated at room temperature by the following procedure. Modified enzyme in 50 mM NH_4HCO_3 was denatured by addition of solid urea to a final concentration of 4 M, and dithiothreitol was added in a 10-fold molar excess over the number of moles of sulfhydryl groups in the enzyme. After 1 h, iodoacetic acid was added in a 2-fold excess over the number of moles of dithiothreitol added previously, followed 10 min later by the addition of β-mercaptoethanol in a 10-fold excess over the moles of iodoacetic acid. After an additional 10-min incubation, the modified enzyme was dialyzed overnight at 4 °C against 50 mM NH_4HCO_3.

Proteolytic Digestion of 5′-SBϵA Modified Enzyme. After carboxymethylation and overnight dialysis, the modified enzyme was successively digested with trypsin and chymotrypsin. Digestion with *N*-tosylphenylalanine chloromethyl ketone–trypsin was carried out at 37 °C with a trypsin/modified enzyme ratio of 1/20 in 50 mM NH_4HCO_3, pH 8.0. The reaction mixture was incubated for 3 h, after which another addition of trypsin in the ratio of 1/20 was made. The incubation was continued at 37 °C for an additional 3 h. At the end of the incubation, the total digest was lyophilized. The lyophilized digest was dissolved in 15 mL of 50 mM NH_4HCO_3, pH 8.0, and maintained at room temperature for 7 h. Chymotrypsin was added in a ratio of 1/20 of the original amount of modified enzyme to the solution of the tryptic digest and incubated at 37 °C for 3 h. After this time, the digest was lyophilized.

Fractionation of Tryptic–Chymotryptic Peptides by Gel Filtration. Aliquots of the total digest (5–10 mg) were dissolved in 0.7 mL of 50 mM NH_4HCO_3, pH 8.0, and applied to a column of Sephadex G-50, mesh 80 (47.4 × 1 cm), equilibrated with 50 mM NH_4HCO_3, pH 8.0, at room temperature. The column was eluted 50 mM NH_4HCO_3, pH 8.0, at a flow rate of 15 mL/h and 0.5-mL fractions were collected. For [^{14}C]SBϵA-labeled peptides, a 20-μL aliquot was removed from each fraction, and the radioactivity was measured in ACS scintillation liquid (Amersham) with a Packard TriCarb liquid scintillation counter, Model 3330. Fractions were pooled on the basis of radioactivity and absorbance at 280 nm and were lyophilized. In order to hydrolyze the ester linkage between the ethenoadenosine and benzoyl moieties, the peptides were resuspended in 1.0 mL of 0.1 M NH_4OH, incubated at room temperature for 4 h, and lyophilized.

Separation of Fractionated Peptide Pools by Reverse-Phase High-Performance Liquid Chromatography. Peptides were separated on a Varian Model 5000 liquid chromatography system equipped with a Varian C_{18} MCH-10 (30 cm × 4 mm) analytical high-performance liquid chromatograph column. Peptides were dissolved in 0.5 mL of 50 mM NH_4HCO_3, pH 8.0, and filtered through 0.2-μm membrane filters (Schleicher & Schuell) prior to injection. Initial separation of peptides was carried out by elution with 1% NH_4HCO_3, pH 7.8, from 0 to 10 min followed by a linear gradient of acetonitrile of 0 to 60% from 10 to 130 min with a flow rate of 1 mL/min. The UV absorbance of the effluents was monitored at 220 nm, and fractions were collected. When radioactive-labeled peptides were separated, an aliquot (50 μL) was removed and assayed

for radioactivity. Peak fractions were pooled and lyophilized. Secondary HPLC runs for the purification of peptides used 0.1% trifluoroacetic acid, pH 2.0, from 0 to 10 min followed by a linear gradient of 0 to 60% (0.08% trifluoroacetic acid in acetonitrile) from 10 to 130 min. The flow rate was 1 mL/min. Prior to lyophilization of desired peptide peaks, the 1-mL fractions were adjusted to pH 7.3 by the addition of 0.17 mL of 0.1 M NH_4OH to prevent decomposition of the carboxybenzenesulfonyl-modified tyrosine and lysine residues.

Amino Acid Analysis of Peptides. Peptides (2–6 nmol) were hydrolyzed in 6 N HCl (after flushing with nitrogen) at 110 °C for 22 h in evacuated, sealed tubes. The hydrolysate was dried, diluted in 0.2 N sodium citrate, pH 2.2, and applied to a single column system, Beckman Model 119C amino acid analyzer. The following buffer change schedule was followed: initial buffer was 0.2 N sodium citrate pH 3.25, for 57 min; buffer was changed to 0.2 N sodium citrate, pH 4.25, for 15 min; finally, the buffer was changed to 0.2 N sodium citrate, pH 6.4, from 72 to 170 min. Under these conditions, CBS-Lys and CBS-Tyr elute at 96 and 108 min, respectively. For comparison, the elution time of leucine was 94 min, tyrosine, 101 min, phenylalanine, 105 min, and histidine, 116 min. For hydrolysates of radioactive peptides, 1.0-min fractions (0.90 mL) of the effluent were collected from the amino acid analyzer after passage through the photometer. Fractions were assayed by adding 0.1 mL of concentrated HCl (12.1 N) to each fraction prior to the addition of 10 mL of ACS scintillation liquid (Amersham).

The level of amino acid contamination was determined after acid hydrolysis and amino acid analysis of fractions obtained from a blank run of 1% NH_4HCO_3/acetonitrile on the HPLC. Fractions were collected corresponding to regions where the modified peptides were detected. The contaminant amino acids detected were aspartic acid, threonine, serine, glutamic acid, glycine, and alanine. The amount of amino acids measured in the peptide samples were corrected for the low level of background contamination. An average of four blank runs was used to correct the amounts of amino acids in the peptide samples.

N-Terminal Analysis. The amino-terminal end of a purified peptide (0.2–0.5 nmol) was labeled with dansyl chloride by using the procedure described by Gray (1972). The dansyl amino acid was identified by two-dimensional thin-layer chromatography on 5 × 5 cm micropolyamide sheets (Schleicher & Schuell) in the solvent systems described by Gray (1972).

Results and Discussion

Comparison of Radioactive and Nonradioactive 5′-SBεA-Labeled Peptides. The isolation and identification of the 5′-SBεA-labeled peptides was carried out on both radioactive and nonradioactive 5′-SBεA-labeled peptides. Glutamate dehydrogenase modified with both types of compounds was analyzed because the amount of [^{14}C]-5′-FSBεA was limited by the low yields from the synthesis of *p*-fluorosulfonyl-[^{14}C]benzoyl chloride (Esch & Allison, 1978); on the average, the overall yield of [^{14}C]-5′-FSBεA from the 0.5 mCi of *p*-amino[^{14}C]benzoic acid starting material was 3%. The modification of glutamate dehydrogenase with 5′-FSBεA requires large amounts of reagent due to the relatively high K_D for reaction (1.4 mM) and the necessity for successive additions of reagent to achieve total modification because of reagent decomposition (Jacobson & Colman, 1982). In contrast to [^{14}C]-5′-FSBεA, the nonradioactive 5′-FSBεA is synthesized by direct coupling of commercially available *p*-fluorosulfonylbenzoyl chloride with ethenoadenosine; therefore, the amount of unlabeled 5′-FSBεA could be prepared in greater quantities, and larger amounts of modified enzyme were prepared with the nonradioactive compound. The [^{14}C]-5′-SBεA modified enzyme was used to locate the 5′-SBεA-labeled peptides among the unlabeled peptides in separations by high-performance liquid chromatography and to determine whether the labeled peptides contained CBS-tyrosine or CBS-lysine residues. The nonradioactive preparations of 5′-SBεA-modified enzyme used to provide adequate amounts of peptides for definitive amino acid analyses of purified peptides. The results presented and discussed represent a composite of the analyses of radioactive and nonradioactive peptides.

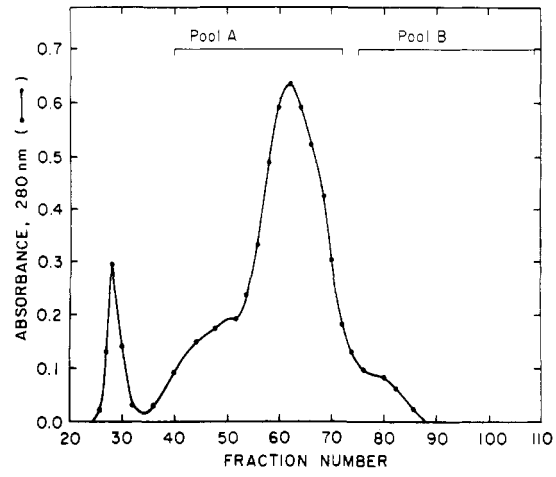

FIGURE 1: Fractionation of tryptic–chymotryptic peptides on Sephadex G-50-80. An aliquot of the total tryptic–chymotryptic digest (5 mg) was applied to the column equilibrated with 50 mM NH_4HCO_3, pH 8.0. The void volume was 14 mL, and total column volume was 37.3 mL. Elution was carried out with 50 mM NH_4HCO_3. Fractions (0.5 mL) were collected and monitored for absorbance at 280 nm.

Fractionation of Tryptic–Chymotryptic Peptides of 5′-SBεA-Modified Enzyme. Aliquots of the total tryptic–chymotryptic digest were first fractionated on a Sephadex G-50-80 column as described under Experimental Procedures. The elution profile as measured by the absorbance at 280 nm is shown in Figure 1. Fractions were grouped into pool A and B, as indicated. The majority of the peptides were found in pool A (fractions 40–72); this pool exhibited the relatively low ratio of 3.8×10^3 cpm per absorbance unit at 280 nm. A shoulder of absorbance (pool B) was observed, which eluted in a volume exceeding the total column volume (fractions 75–108); a substantial amount of radioactivity was associated with this pool B, with relatively little absorbance at 280 nm, as reflected in the much higher ratio of abount 4.7×10^4 cpm/A_{280nm} unit. The CBS-Tyr-containing peptides were located predominantly in pool B, and this pool was purified further. The retention of pool B relative to pool A may be due to hydrophobic interactions between the Sephadex matrix and the 5′-SBεA label of the pool B peptides.

In order to hydrolyze the ester linkage between the ethenoadenosine and benzoyl moieties, the 5′-SBεA peptides of pool A and pool B were treated with base as described under Experimental Procedures. This procedure was employed prior to HPLC separation to convert the labeled peptides into a single form containing carboxyphenylsulfonyl-modified residues without the ethenoadenosine moiety. The modified peptides will subsequently be referred to as CBS-peptides, indicating that the ethenoadenosine group is not present. It was anticipated that conversion of the modified peptides to a single form would simplify the separation analysis, and the hydrolysis of the ester linkage between ethenoadenosine and benzoyl moieties might decrease the hydrophobicity of the

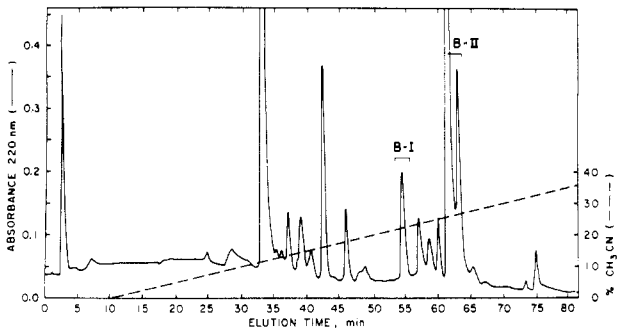

FIGURE 2: Separation of pool B peptides on reverse-phase high-performance liquid chromatography in ammonium bicarbonate. Pool B from the Sephadex G-50-80 column was dissolved in 50 mM NH_4HCO_3 and applied to a Varian C_{18} MCH-10 column. Peptides were eluted with 1% NH_4HCO_3, pH 7.8, from 0 to 10 min, followed by a linear gradient of acetonitrile, as described under Experimental Procedures. Absorbance (—) was monitored at 220 nm. The linear gradient of acetonitrile is indicated by the dashed line (---). Peptide peaks B-I (fractions 54–55) and B-II (fractions 62–64) were pooled as indicated and lyophilized. This run represents pool B resulting from the fractionation of 10 mg of the original total digest.

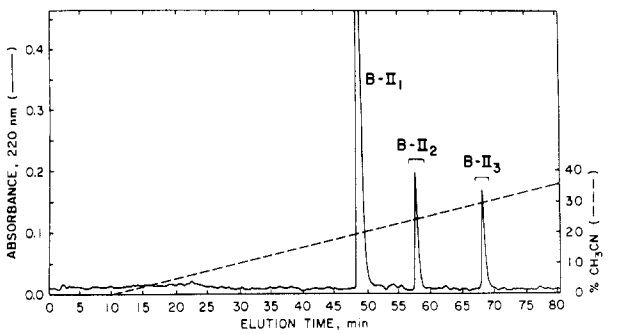

FIGURE 3: Purification of B-II on reverse-phase high-performance liquid chromatography in trifluoroacetric Acid. B-II pooled from three separations of pool B peptides in 1% NH_4HCO_3 (Figure 2) was rechromatographed in 0.1% trifluoroacetic acid, ph 2.0, with a linear gradient of 0.08% trifluoroacetic acid in acetonitrile as described under Experimental Procedures. Absorbance was monitored at 220 nm (—). The linear gradient of acetonitrile is indicated by the dashed line (---).

labeled peptides by removing the ethenoadenosine group.

HPLC Separation of Pool B Tryptic–Chymotryptic Peptides. Pool B peptides isolated from the Sephadex G-50-80 column and base hydrolyzed were separated by HPLC in 1% NH_4HCO_3, pH 7.8, with linear gradients of acetonitrile as described under Experimental Procedures. The total pool B was separated by three successive HPLC runs which exhibited identical elution patterns, as illustrated by Figure 2. No additional UV-absorbing peaks were observed above 35% acetonitrile. Two major peaks of radioactivity were detected in fractions 54–55 (B-I) and 62–64 (B-II) which correspond to 22% acetonitrile and 27% acetonitrile, respectively. The percent radioactivity recovered in B-I and B-II relative to the total amount of counts injected into the HPLC was 38% for B-I and 29% for B-II. Smaller amounts of radioactivity found in fractions 33–34 and fraction 37 represented 12% and 19%, respectively, of the total radioactivity applied to the HPLC. Peak B-I was judged to be a pure peptide because of the symmetrical appearance of the peak and its good separation from the other peaks. Purification of B-II required a secondary HPLC run, and the trifluoroacetic acid/acetonitrile solvent system described under Experimental Procedures was utilized. The rerun of B-II in trifluoroacetic acid/acetonitrile was carried out on nonradioactive CBS-labeled peptides fractionated by the same procedures used for ^{14}C-labeled peptides. The HPLC pattern in 1% NH_4HCO_3 of the nonradioactive CBS-labeled peptides was similar to that observed for the [^{14}C]-CBS-labeled peptides. The purification of B-II is shown in Figure 3. Three well-resolved peaks in fractions 48–50 (B-II_1), fractions 57–59 (B-II_2), and fractions 67–69 (B-II_3) corresponding to 20%, 24%, and 29% acetonitrile, respectively, were observed. No other UV-absorbing peaks were observed above 35% acetonitrile. Because the B-II pool utilized for the secondary HPLC separation did not contain a radioactive CBS label, the carboxyphenylsulfonyl-labeled peptide among these three peaks was identified by a different approach. Peak B-II_1 was determined to be the larger UV-absorbing contaminant corresponding to fraction 61 of the initial HPLC separation of pool B in 1% NH_4HCO_3 (Figure 2); a single peak corresponding to B-II_1 was observed when fraction 61 from the initial separation in 1% NH_4HCO_3 was rechromatographed in the trifluoroacetic acid/acetonitrile system. Peaks B-II_2 and B-II_3 were pooled separately, lyophilized, and subjected to amino acid analysis. No CBS-Lys or CBS-Tyr was detected in B-II_3; however, CBS-Tyr was detected in the amino acid analysis of B-II_2, and this peptide was therefore identified as one of those modified by 5′-FSBεA.

Table I: Comparison of the Amino Acid Compositions of B-I and B-II_2 Peptides

	peptide B-I		peptide B-II_2	
amino acid	amount measured[a] (nmol)	mol/mol of peptide[b]	amount measured[a] (nmol)	mol/mol of peptide[b]
Asp	0.43	0.08 (0)	0.22	0.09 (0)
Thr	0.02	0.00 (0)	–	–
Glu	0.07	0.01 (0)	–	–
Gly	1.08	0.21 (0)	–	–
Ala	0.31	0.06 (0)	–	–
Lys	0.62	0.12 (0)	–	–
Leu	5.38	1.06 (1)	2.69	1.08 (1)
His	3.72	0.73 (1)	2.22	0.90 (1)
Arg	5.09	1.00 (1)	2.48	1.00 (1)
CBS-Tyr	3.55	0.70 (1)	1.75	0.71 (1)

[a] The amount of each amino acid measured was corrected for a low level of background contamination as described under Experimental Procedures. The dash (–) indicates that the amino acid was not detected in the amino acid analysis in amounts greater than that in the blanks. In addition, Ser, Pro, Val, Met, Ile, Tyr, and Phe were not detected. [b] The amount of each amino acid measured was normalized to arginine.

Amino Acid Composition of B-I and B-II_2. The amino acid compositions of B-I and B-II_2 are compared in Table I. Both peptides contained CBS-Tyr and the amino acid compositions appear identical (Table I, bottom). The actual amount of each amino acid measured was normalized to the amount of arginine since it is probable that arginine is the carboxy-terminal residue of the peptide based on the specificity of cleavage by trypsin. Radioactive B-I and B-II_2 were also hydrolyzed and subjected to amino acid analysis. CBS-Tyr was detected in the amino acid elution profile, and radioactivity was measured in the fractions collected from the amino acid analyzer corresponding to the CBS-Tyr region (108 min). The nanomoles of CBS-Tyr measured from the radioactivity was in good agreement with that calculated from the ninhydrin reaction by comparison with the color constant for a CBS-Tyr standard: for B-I, 1.7 nmol of CBS-Tyr was measured from the ninhydrin positive peak in comparison to 1.9 nmol of CBS derivative determined from the measurement of radioactivity prior to application to the analyzer; for B-II_2, 1.4 nmol of CBS-Tyr was measured from a ninhydrin positive peak in comparison to 1.9 nmol of CBS derivative determined from the measurement of radioactivity prior to application to the

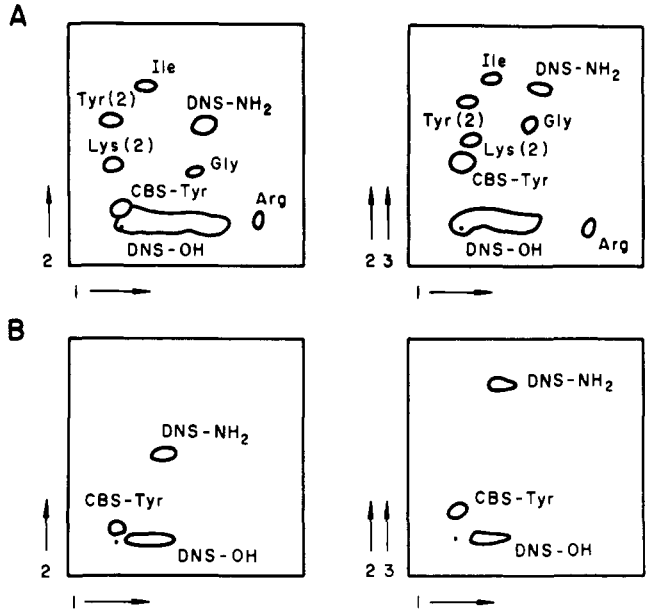

FIGURE 4: Two-dimensional thin-layer chromatography of dansylated CBS-Tyr and peptide B-I. Samples were dansylated following the procedure described under Experimental Procedures. Fluorescent spots were identified by two-dimensional thin-layer chromatography on polyamide sheets. The numbers and arrows indicate which solvent system and the development direction, respectively; 1 = water–90% formic acid (200:3), 2 = benzene–acetic acid (9:1), and 3 = ethyl acetate–methanol–acetic acid (20:1:1). The black dot in the lower left corner of each square indicates the origin. (A) Chromatography of dansylated CBS-Tyr standard; the positions of some standard dansyl amino acids are also indicated for reference; Tyr (2) and Lys (2) are abbreviations for O,N^{α}-didansyltyrosine and N^{α},N^{ϵ}-didansyllysine; DNS-OH is dansylic acid; DNS-NH_2 is dansylamide. (B) Chromatography of dansylated B-I. (A similar chromatogram was obtained for B-II_2.)

amino acid analyzer. The regions of the HPLC separation of pool B (Figure 2) which contained small amounts of radioactivity, fractions 33–34 and 37, were also analyzed for the presence of CBS-Tyr or CBS-Lys. Fractions collected from the amino acid analyzer contained radioactivity only in the region corresponding to an elution time of 14 min. For comparison, aspartic acid elutes at 24 min. A possible decomposition product of CBS-Lys is *p*-sulfonylbenzoic acid which results from the hydrolysis of the sulfonamide linkage (Likos et al., 1980). In contrast, CBS-Tyr is stable toward acid hydrolysis. A ninhydrin negative peak of radioactivity for this product elutes around 13 min from an amino acid analyzer (J. J. Likos, and R. F. Colman, unpublished results), in agreement with the radioactivity detected here at an elution time of 14 min. It is possible that the small amounts of radioactivity detected in these minor peaks may be attributed to modified lysine-containing peptides.

N-Terminal Analysis of B-I and B-II_2. The amino-terminal residue of peptides B-I and B-II_2 was determined after labeling with dansyl chloride and conducting two-dimensional thin-layer chromatography on polyamide sheets as described under Experimental Procedures. A standard solution of CBS-Tyr was dansylated and analyzed on the polyamide sheets. Dansyl-CBS-Tyr was identified as a yellow fluorescent spot which remains relatively close to the origin after development in two dimensions (Figure 4A, left square). This spot migrated with a significantly higher R_f after development in the third solvent, as indicated in Figure 4A (right square). The migration of dansylated CBS-Tyr differs from all other dansylated amino acids, allowing its identification readily. CBS-Tyr was identified as the amino-terminal residue after labeling peptides B-I and B-II_2 with dansyl chloride, as illustrated by Figure 4B. No other fluorescent spots in addition to CBS-Tyr were observed for B-I and B-II_2, except for dansylamide and dansylic acid. This result further demonstrates the purity of these peptide peaks and indicates that both of these peptides have CBS-Tyr as an amino-terminal residue.

Peptides B-I and B-II_2 are the only peptides in which CBS-tyrosine was detected. Therefore, 5′-FSBϵA appears to label a unique tyrosine in glutamate dehydrogenase.

Relationship of Peptide Peaks B-I and B-II_2. The similarity of the compositions of peptides B-I and B-II_2 and the demonstration of CBS-Tyr as the amino-terminal residue for both peptides provide evidence that these peptides are identical even though they elute as distinct peaks at different compositions of acetonitrile (22% for B-I in comparison to 27% for B-II) in the initial separation of pool B peptides in 1% NH_4HCO_3. In order to establish the relationship between peptides B-I and B-II_2, both peaks were isolated and rechromatographed separately by HPLC in 1% NH_4HCO_3 under the conditions of Figure 2. Each sample gave a single peak at 27% acetonitrile, corresponding to the elution position of B-II of the original run. Others have also observed single peptides that give two peaks, apparently due to an artifact of the separation procedure [e.g., see Hollemans et al. (1983)]; this may be the result of a differential interaction of the ionic species of the NH_4HCO_3 solution with the peptide.

Sequence of CBS-Tyr Peptides. The primary sequence of glutamate dehydrogenase (Julliard & Smith, 1979) was examined for the occurrence of a sequence which coincided with the amino acid composition determined for B-I and B-II_2 and agreed with the expected cleavage pattern generated from the digestion with trypsin and chymotrypsin. The peptide fragment

$$Tyr^{262}\text{-Leu-His-}Arg^{265}$$

contains the four amino acids determined from the compositional analysis of B-I and B-II_2, has tyrosine for an amino-terminal residue, and would be an expected peptide fragment resulting from a digestion with trypsin and chymotrypsin; the residue preceding tyrosine-262 is phenylalanine. In addition, peptide 262–265 is the only region in the primary sequence where histidine occurs relatively close (within two residues) to *both* tyrosine and arginine. Tyrosine-262 is the only tyrosine residue in glutamate dehydrogenase which is modified by 5′-FSBϵA, and it is concluded that this residue is the essential tyrosine involved in the inhibition site of glutamate dehydrogenase.

HPLC Separation of Pool A Tryptic–Chymotryptic Peptides. Pool A peptides isolated from the Sephadex G-50-80 column were separated by HPLC in 1% NH_4HCO_3, pH 7.8, with linear gradients of acetonitrile as described under Experimental procedures. Peaks of radioactivity were observed at 12.5% acetonitrile (designated A-I), at 35% acetonitrile (designated A-II), and at 27% acetonitrile. The peak at 27% acetonitrile corresponded in elution position with B-II, indicating that some overlap of the pool B peptides occurs in pool A. CBS-Lys was the only modified amino acid found in A-I and A-II. A-I and A-II were not pure peptides; however, the two pools did not include the same amino-terminal residues, as determined by dansylation. It is tentatively considered that at least two different lysine residues are modified by 5′-FSBϵA.

Conclusion

The specific peptides of glutamate dehydrogenase modified by 5′-FSBϵA have been isolated in the attempt to relate specific residues of the primary sequence to particular functions. As

a result of modification by 5′-FSBεA, the enzyme exhibits an altered affinity for and a diminished extent of inhibition by saturating concentrations of GTP. From direct binding studies, it has been shown that the modified enzyme binds only 1 mol of GTP/mol of subunit in the presence of NADH, in contrast with the 2 mol of GTP/mol of subunit bound by native enzyme, suggesting that one of the GTP sites has been eliminated as a result of reaction with 5′-FSBεA (Jacobson & Colman, 1982). The extent of covalent incorporation (1.28 mol of 5′-SBεA/mol of subunit at maximal change in GTP inhibition) was previously determined to be attributable to 0.95 mol of modified tyrosine/mol of subunit and 0.33 mol of modified lysine/mol of subunit (Jacobson & Colman, 1983). The modification of tyrosine was directly proportional to the change in GTP inhibition and was postulated to be at an essential residue involved in the GTP inhibition. Tyrosine-262 has now been identified as a unique tyrosine residue modified by 5′-FSBεA, and it is concluded that this tyrosine participates in the GTP binding site of glutamate dehydrogenase. In contrast, the modification of lysine by 5′-FSBεA was postulated to occur at a nonessential residue which is not involved in the inhibitory action of GTP. The analysis of CBS-Lys-containing peptides suggests that there are at least two peptides, and therefore, a unique lysine residue is not modified by 5′-FSBεA, as might have been expected from the observed lack of correlation between the extent of lysine modification and the change in GTP inhibition described previously (Jacobson & Colman, 1983).

The implication of tyrosine as the site of modification is not the first report of the involvement of tyrosine in the GTP site. 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 residue has been isolated and the residue identified as tyrosine-407. The tyrosine modified by tetranitromethane is clearly a different residue in the primary sequence of glutamate dehydrogenase than the tyrosine modified by 5′-FSBεA. It is possible that two distinct tyrosines could be involved in the binding of nucleotide to the GTP inhibitory site and that their role in binding nucleotide could be different. The guanine portion of GTP would probably exhibit preference for interaction with a hydrophobic binding region within the enzyme, and it could be postulated that this binding is stabilized by stacking interactions with an aromatic residue such as tyrosine. A role for tyrosine involvement in the binding of the phosphate moieties of GTP might also be suggested: a bonding interaction could occur between the phenolic hydrogen of tyrosine and one of the ionized phosphates of the polyphosphate chain. Therefore, two different tyrosines could participate simultaneously in the binding of GTP, and these residues might exhibit differential reactivity or availability to the reagents tetranitromethane and 5′-FSBεA.

The conformation of enzyme-bound 5′-FSBεA has recently been evaluated by measuring the quantum yield of the analogue when enzyme bound and comparing that value to the quantum yield of free 5′-FSBεA in various solvents (Jacobson & Colman, 1984). The quantum yield of covalently bound 5′-SBεA did not differ significantly from the relatively low quantum yield for free 5′-FSBεA in aqueous solution, indicating that the analogue exists in a conformation of the enzyme in which the purine base and the benzoyl group are stacked. From these results, it can be inferred that the amino acid residue specifically modified by 5′-FSBεA is located in the purine binding region of the enzyme site rather than the terminal phosphate binding site. It is proposed that the essential tyrosine modified by 5′-FSBεA on glutamate dehydrogenase is involved in the binding of the guanine moiety of GTP and the region of the enzyme primary sequence around tyrosine-262 may contribute to the binding of the purine moiety of GTP.

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Registry No. 5′-FSBεA, 76021-83-5; 5′-FSBεA labeled, 93255-03-9; GTP, 86-01-1; L-tyrosine, 60-18-4; glutamate dehydrogenase, 9029-12-3; ethenoadenosine, 39007-51-7; *p*-(fluorosulfonyl)[^{14}C]benzoyl chloride, 65937-32-8.

References

Colman, R. F. (1983) *Annu. Rev. Biochem. 52*, 67–91.

Esch, F. S., & Allison, W. S. (1978) *Anal. Biochem. 84*, 642–645.

Goldin, B. R., & Frieden. C. (1972) *Curr. Top. Cell. Regul. 4*, 77–117.

Gray, W. R. (1972) *Methods Enzymol. 25*, 121–138.

Hollemans, M., Runswick, M. J., Fearnley, I. M., & Walker, J. E. (1983) *J. Biol. Chem. 258*, 9307–9313.

Jacobson, M. A., & Colman, R. F. (1982) *Biochemistry 21*, 2177–2186.

Jacobson, M. A., & Colman, R. F. (1983) *Biochemistry 22*, 4247–4257.

Jacobson, M. A., & Colman, R. F. (1984) *J. Biol. Chem. 259*, 1454–1460.

Julliard, J. H., & Smith, E. L. (1979) *J. Biol. Chem. 254*, 3427–3438.

Likos, J. J., Hess, B., & Colman, R. F. (1980) *J. Biol. Chem. 255*, 9388–9398.

Olson, J. A., & Anfinsen, C. B. (1952) *J. Biol. Chem. 197*, 67–79.

Pal, P. K., & Colman, R. F. (1979) *Biochemistry 18*, 838–845.

Pantaloni, D., & Dessen, P. (1969) *Eur. J. Biochem. 11*, 510–519.

Penefsky, H. S. (1979) *Methods Enzymol 56*, 527–530.

Piszkiewicz, D., Landon, M., & Smith, E. L. (1971) *J. Biol. Chem. 246*, 1324–1329.

Price, N. C., & Radda, G. K. (1969) *Biochem. J. 114*, 419–427.

Saradambal, K. V., Bednar, R. A., & Colman, R. F. (1981) *J. Biol. Chem. 256*, 11866–11872.

Smith, E. L., & Piszkiewicz, D. (1973) *J. Biol. Chem. 248*, 3089–3092.

Smith, E. L., Landon, M., Piszkiewicz, D., Brattin, W. J., Langley, J. J., & Malamed, M. D. (1970) *Proc. Natl. Acad. Sci. U.S.A. 67*, 724–730.