Identification of the Lysine and Tyrosine Peptides Labeled by 5'-p-Fluorosulfonylbenzoyladenosine in the NADH Inhibitory Site of Glutamate Dehydrogenase*

Jane A. Schmidt and Roberta F. Colman
From the Department of Chemistry, University of Delaware, Newark, Delaware 19716

In contrast, the affinity label 5'-FSBA reacts specifically at the NADH inhibitory site of glutamate dehydrogenase (8). After reaction with 5'-FSBA, the enzyme retains full catalytic activity in the absence of allosteric ligands and is still sensitive to activation by ADP and to inhibition by GTP. However, the modified enzyme is no longer inhibited by NADH above 10^{-4} M. High concentrations of NADH alone or NADH plus GTP protect glutamate dehydrogenase against reaction with 5'-FSBA, whereas ADP or GTP alone do not. Moreover, the incorporation of ^3H-labeled reagent into the protein is proportional to the decreased inhibition by high concentrations of NADH, reaching 0.53 sulfonfonylbenzoyladenosine/subunit or 3 sulfonylbenzoyladenosine/hexameric enzyme when NADH inhibition is completely lost. After acid hydrolysis, all of the incorporated reagents/subunit can be accounted for by 0.28 mol Tyr-(CBS) and 0.25 mol Lys-(CBS) (9). Since the ratio of these two modified amino acids remains constant throughout the modification reaction, the lysine and tyrosine residues of glutamate dehydrogenase which react with 5'-FSBA both appear to be located in the NADH inhibitory site. Modification of either residue on three subunits of the hexameric enzyme is sufficient to eliminate inhibition by NADH.

In this paper, we report the isolation of the peptides containing the modified residues and the identification of the specific lysine and tyrosine which react with 5'-FSBA. A preliminary account of this work has been presented (10).

EXPERIMENTAL PROCEDURES

Isolation of 5'-FSBA-modified Peptides—It was previously reported by Annamalai et al. (17) that dihydroxyboryl-substituted polyacrylamide or cellulose columns could be used to purify peptides modified with fluorosulfonylbenzoyl nucleotide analogues. The modified peptides are bound to such columns by the reversible interaction of the cis-diol moiety of the reagents with the column-bound boronate. We have used a PBA column to achieve a large initial purification of the 5'-

---

1 The abbreviations used are: 5'-FSBA, 5'-p-fluorosulfonylbenzoyladenosine; Lys-(CBS), N-(4-carboxybenzenesulfonyl)lysine; Tyr-(CBS), O-(4-carboxybenzenesulfonyl)tyrosine; HPLC, high-performance liquid chromatography; PBA, phenyl boronate-agarose; SBA, sulfonylbenzoyladenosine.

Portions of this paper (including "Experimental Procedures," and Figs. 1-4) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9850 Rockville Pike, Bethesda, MD 20814, Request Document No. 84M-1977, cite the authors, and include a check or money order for $2.80 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
FSBA-modified peptides of glutamate dehydrogenase (Fig. 1). The majority of the peptides in the thermolysin digest of 5'-FSBA-modified glutamate dehydrogenase are not bound to the PBA column and are removed by an initial wash with 50 mM phosphate buffer, pH 7.8. Very little of the modified peptides were eluted in this wash, as evidenced by the small amount of radioactivity in these fractions. The column was then washed with deionized water to elute the nucleosidyl peptides. These peptides eluted in a single sharp peak; the absorbance at 220 nm coincided with the 3H counts. Since the radioactivity trailed, a broad pool from the peak through the rest of the H2O wash was collected. The yield of modified peptides was 54–68% of the amount applied to the column, based on the radioactivity. A final wash of the column with 0.1 M ammonium acetate, pH 5.2, removed a small amount of [3H]adenosine which presumably resulted from the hydrolysis of a small portion of the peptide-bound reagent. [3H]Adenosine accounted for only about 10–15% of the applied radioactivity.

Annamalai et al. (17) found that the water eluate from phenyl boronate columns contained only nucleosidyl peptide(s). However, when we applied a much larger amount of digested protein (40–50 mg versus 9–10 mg) to the PBA column, we found that the water fractions contained a larger number of peptides than expected. It is likely that the unmodified peptides were retained on the column by hydrophobic interactions with the column-bound phenyl groups. For this reason, and also to separate the expected lysine-containing and tyrosine-containing nucleosidyl peptides, it was necessary to purify the water eluate from the PBA column.

Fractionation of Peptides by HPLC—The peptides in the water eluate from the PBA column were concentrated by lyophilization and then fractionated by HPLC in System B, yielding four major peaks of tritium radioactivity (Fig. 2). Pool a is [3H]adenosine from the hydrolysis of the ester linkage of the reagent. Peak a elutes at 15 min, as does authentic adenosine when chromatographed under the same conditions. Of the applied radioactivity, 5–15% of the counts eluted as [3H]adenosine.

Pool b contained 10–25% of the applied 3H counts, but apparently not in a peptide-bound form. Pool b gave no detectable product with dansyl chloride and, after acid hydrolysis, showed only glycine and trace amounts of a few other amino acids. After base hydrolysis in 0.1 N NaOH for 4 h, pool b ran as adenosine on HPLC on System T. Therefore, we suspected that b represented another product from the breakdown of the reagent. The fluorosulfonyl moiety of 5'-FSBA can be hydrolyzed to F- and a sulfonic acid by incubation at pH 8.0 (18). We allowed 5'-FSBA to undergo such hydrolysis and monitored the reaction over a period of 5 h by HPLC in System B. 5'-FSBA eluted at 41 min and authentic adenosine at 15 min. With time, the peak of 5'-FSBA decreased, and a new peak developed at 17 min, coincident with the peak of pool b in Fig. 2. Thus, b is the sulfonic acid of the reagent from the decomposition of the labeled peptides.

Pools c and d of Fig. 2 contained 10–20% and 10–25%, respectively, of the applied radioactivity. Each pool was lyophilized and then treated with 0.1 N NaOH for 4 h at room temperature to completely hydrolyze the ester linkage of the reagent to form [3H]adenosine and CBS-peptides. After base hydrolysis, c and d were neutralized with acetic acid and separately chromatographed by HPLC in System T. Pool d (Fig. 3) gave a large peak of [3H]adenosine at 6 min, and a single large peak of a CBS-peptide at 26 min, in addition to at least two other small peaks of contaminating peptides. Pool c (Fig. 4) also gave a large peak of [3H]adenosine and a peak of CBS-peptide at 21 min, plus several small peaks of contaminants. The two major peaks of CBS-peptides (I and II) were collected as indicated by the type in Fig. 3 and Fig. 4, respectively.

Analysis of the Isolated Peptides—Peaks I and II were dried in vacuo, then resuspended in water and dried again several times to remove trifluoroacetic acid. A portion of each was treated with dansyl chloride for NH2-terminal analysis, and the majority was hydrolyzed for amino acid analysis. The results are summarized in Table I. The amount of Lys-(CBS)-modified peptide in peak I is low compared to the amounts of Glx, Leu, and Arg, but when Lys-(CBS) and Lys are considered together, there is a comparable amount. Saradambal et al. (9) found that 15.5% of Lys-(CBS) was lost on hydrolysis at 110 °C; apparently a much greater portion of Lys-(CBS) is hydrolyzed to Lys during hydrolysis at 120 °C. When an independently isolated sample of II (IIa, Table I) was hydrolyzed at 110 °C, much less loss of Lys-(CBS) occurred.

From comparison of the data in Table I with the known amino acid sequence of bovine liver glutamate dehydrogenase (7), the isolated peptides are identified as the following unique sequences:

\[
\begin{align*}
I &= \text{H}_2\text{N-Ile-Gly-His-Tyr(CBS)-Asp-COOH} & (\text{Residues 187–191}) \\
II &= \text{H}_2\text{N-Leu-Glu-Arg-Lys(CBS)-COOH} & (\text{peptide II})
\end{align*}
\]

Therefore, the residues modified by 5'-FSBA are Tyr-190 and Lys-420.

DISCUSSION

We have isolated two peptides from the thermolysin digest of glutamate dehydrogenase modified by 5'-fluorosulfonylbenzoyladenosine and identified them as H_2N-Ile-Gly-His-Tyr(CBS)-Asp-COOH (peptide I) and H_2N-Leu-Glu-Arg-Lys(CBS)-COOH (peptide II). The amino acid analysis and NH2-terminal residues in Table I fit uniquely with these two fragments of the known amino acid sequence. Peptide I corresponds to residues 187–191 and peptide II to residues 417–420. 5'-FSBA modifies Tyr-190 and Lys-420.

Lys-420 has been previously modified with trinitrobenzenesulfonate, along with Lys-423 (19). In that case, however, it was Lys-423 whose modification was associated with the loss of inhibition of the enzyme by excess NADH. Our results with the affinity labeling reagent 5'-FSBA for glutamate dehydrogenase are more specifically with glutamate dehydrogenase than does trinitrobenzenesulfonate, clearly indicate that Lys-420 is part of the NADH inhibitory site. We found no indication that Lys-423 was also modified by 5'-FSBA, suggesting that Lys-423 is in a region of the NADH inhibitory site not accessible to 5'-FSBA.

The reaction of 5'-FSBA with glutamate dehydrogenase is limited and specific. The major effect of the modification is the loss of sensitivity of the enzyme to inhibition by high concentrations of NADH. Allosteric regulation by ADP and GTP are largely unaffected, and the enzyme retains full catalytic activity (8). Furthermore, NADH, but not ADP or GTP, protects the enzyme against the loss of NADH inhibition (8, 9). Modified glutamate dehydrogenase contains only 0.53 sulfonylbenzoyladenosine/subunit, and the label is distributed approximately equally between modified lysine and tyrosine (9). Thus, the modified residues, Tyr-190 and Lys-420, must both be located in or near the NADH inhibitory site of the enzyme. The modification of equal amounts of these two residues suggests that both are close to the reactive sulfonylfluoride of the enzyme-bound reagent and have an equal probability of reacting. Alternatively, it may indicate...
that the reagent binds to the protein in two distinct, but equally probable, conformations that bring the sulfonylfluoride close to one or the other reactive residue. While we cannot conclusively demonstrate which model of 5'-FSBA binding is correct, the great specificity of the reaction of the affinity label with glutamate dehydrogenase argues for a unique mode of binding with the reactive moiety close to both Lys-420 and Tyr-190. Thus, although these two residues are separated by almost half the protein in the linear sequence, they appear to be quite close to each other in the folded structure.

If 5'-FSBA binds to the NADH inhibitory site of glutamate dehydrogenase in a conformation similar to that which bound NADH assumes, then the reactive sulfonylfluoride moiety occupies the subsite for the nicotinamide ribose or possibly that for the pyrophosphate. It is easy to envision roles for lysine and tyrosine residues in those subsites. The ribose moieties of NAD are bound to dehydrogenases by hydrogen bonds and hydrophobic interactions, while the pyrophosphate moiety is bound by electrostatic interactions (20). Lys-420, unprotonated, and a ribose hydroxyl could form a hydrogen bond, or a protonated lysine could participate in an electrostatic interaction with the pyrophosphate of NADH. The role of Tyr-190 could involve hydrophobic interaction with the nicotinamide ribose or hydrogen bonding with the ribose hydroxyls or the pyrophosphate oxygens.

Wooton (21) and Austen et al. (22) have applied rules for secondary structure prediction to the sequence of glutamate dehydrogenase to attempt to find dinucleotide binding domains, similar to the generalized NAD-binding region described by Rossmann (23). Two such domains were predicted, formed by residues 9–128 and by residues 245–356. Neither of the predicted NADH binding domains include Tyr-190 or Lys-420, which we have identified as being part of the inhibitory site. Rossmann described the dinucleotide binding domain based on the x-ray crystal structures of several dehydrogenases with NAD or NADP bound at their active sites. The NADH site of glutamate dehydrogenase which is labeled by 5'-FSBA is an allosteric site and is apparently not closely analogous to the catalytic binding sites of other dehydrogenases.

Spectroscopic studies of the binding of NADH to glutamate dehydrogenase have indicated that the adenine portion of NADH is of primary importance for binding to the inhibitory site (6, 24). This is also reflected in the affinity labeling of that site by the analog 5'-FSBA, which lacks the nicotinamide portion entirely.

Incorporation of 0.53 sulfonylbenzoyladenosine/subunit indicates that modification of three subunits of the hexameric enzyme is sufficient to eliminate NADH inhibition. Glutamate dehydrogenase has exhibited half-site effects in several other types of experiments. CD studies indicated a conformational transition in the enzyme half-saturated with NAD or NADP (25); in the presence of GTP, half-saturation with NADH completely dissociated aggregated forms of the enzyme to hexamers (26); and modification of Lys-126 with pyridoxal 5'-phosphate on two to three subunits of the hexameric enzyme led to loss of NADH inhibition (27), as did modification of Lys-423 last three studies all reflected the binding of NADH at the inhibitory site (6, 24), and cross-linking studies (28) have indicated that the hexameric enzyme is composed of two sets of three subunits. Such an arrangement may provide a structural basis for interactions between subunits.

Our laboratory has recently reported the synthesis of a new purine nucleotide analog 6-((4-bromo-2,3-dioxobutyl)thioa-adenosine 5'-diphosphate (29), in which the reactive moiety is attached to the adenine ring. This affinity-labeling reagent also reacts with glutamate dehydrogenase to label the NADH inhibitory site (30). Since this new reagent presumably labels a reactive amino acid within the adenine-binding subsite, identification of the labeled residue will give additional information about the three-dimensional structure of glutamate dehydrogenase.
dehydrogenase in the region of its NADH inhibitory site. This work is currently in progress.

REFERENCES
1. Krause, J., Bühner, M., and Sund, H. (1974) Eur. J. Biochem. 41, 593–602
2. Sund, H., Markau, K., and Koberstein, R. (1975) Subunits in Biological Systems, Vol. 7c, pp. 225–287, Marcel Dekker, New York
3. Goldin, B. R., and Frieden, C. (1972) Curr. Top. Cell Regul. 4, 77–117
4. Fisher, H. F. (1973) Adv. Enzymol. Relat. Areas Mol. Biol. 39, 269–417
5. Pal, P. K., and Colman, R. F. (1979) Biochemistry 18, 838–845
6. Pantaloni, D., and Dessen, P. (1969) Eur. J. Biochem. 11, 510–519
7. Julliard, J. H., and Smith, E. L. (1979) J. Biol. Chem. 254, 3427–3438
8. Pal, P. K., Wechter, W. J., and Colman, R. F. (1975) J. Biol. Chem. 250, 8140–8147
9. Saradambal, K. V., Bednar, R. A., and Colman, R. F. (1981) J. Biol. Chem. 256, 11866–11872
10. Schmidt, J. A., and Colman, R. F. (1984) Fed. Proc. Fed. Am. Soc. Exp. Biol. 43, 1543
11. Olson, J. A., and Anfinsen, C. B. (1952) J. Biol. Chem. 197, 67–79
12. Smith, E. L., Landon, M., Piszkiewicz, D., Brattin, W. J., Langley, T. J., and Melamed, M. D. (1970) Proc. Natl. Acad. Sci. U.S.A. 67, 724–730
13. Wyatt, J. L., and Colman, R. F. (1977) Biochemistry 16, 1335–1342
14. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
15. Likos, J. J., and Colman, R. F. (1981) Biochemistry 20, 491–499
16. Gray, W. R. (1972) Methods Enzymol. 25, 121–138
17. Annamalai, A. E., Pal, P. K., and Colman, R. F. (1979) Anal. Biochem. 99, 85–91
18. Likos, J. J., Hess, B., and Colman, R. F. (1980) J. Biol. Chem. 255, 9388–9398
19. Goldin, B. R., and Frieden, C. (1971) Biochemistry 10, 3527–3534
20. Grau, U. M. (1982) in The Pyridine Nucleotide Coenzymes (Evresek, J., Anderson, B., and You, K.-S., eds) pp. 135–187, Academic Press, New York
21. Wootton, J. C. (1974) Nature (Lond.) 252, 542–546
22. Austen, B. M., Haberland, M. E., and Smith, E. L. (1980) J. Biol. Chem. 255, 8001–8004
23. Rossmann, M. G., Liljas, A., Bråndén, C.-I., and Banaszak, L. J. (1975) in The Enzymes, (Boyer, P. D., ed) Vol. 11, Part A, pp. 61–102, Academic Press, New York
24. Delabar, J. M., Martin, S. R., and Bayley, P. M. (1982) Eur. J. Biochem. 127, 367–374
25. Bell, J. E., and Dalziel, K. (1973) Biochim. Biophys. Acta 309, 237–242
26. Huang, C. Y. and Frieden, C. (1972) J. Biol. Chem. 247, 3638–3646
27. Goldin, B. R., and Frieden, C. (1972) J. Biol. Chem. 247, 2139–2144
28. Hucho, F., Rasched, I., and Sund, H. (1975) Eur. J. Biochem. 52, 221–230
29. Colman, R. F., Huang, Y.-C., King, M. M., and Erb, M. (1984) Biochemistry 23, 3281–3286
30. Batra, S. and Colman, R. F. (1984) Biochemistry 23, 4940–4946
Lys and Tyr in Inhibitory NADH Site of Glutamate Dehydrogenase

Experimental Procedures

Materials - A crystalline suspension of bovine liver glutamate dehydrogenase, purchased from Boehringer-Mannheim, was dissolved in 0.1 M potassium phosphate buffer, pH 7.1, at a weight of 1%. The dialyzed material was centrifuged to remove precipitated, denatured protein. The concentration of protein was determined from the absorbance at 280 nm using an ε = 1.55 × 10^4 cm^-1 mol^-1. The suspension of 2 ml was used for the molecular weight of the intact subunits (20).

15-S-Fluorophenylthiohydantoin [15-S]-chelosine was prepared according to the procedure of Weathers et al. (1) from [15-S]-chelohaine (New England Nuclear Corp.). The measured specific radioactivity was 1.03 × 10^18 cpm/μmol. The concentration of [15-S]-PSBA was measured fluorometrically, using ε = 1.15 × 10^4 cm^-1 mol^-1 or 1.85 × 10^4 cm^-1 mol^-1 (12).

Thermolyse, thioglycolic acid, and hydrolysis were carried out by Sigma Chemical Co. Phenylboronate agarose, Mono Q 5/5, was from Amersham and Pharmacia Biotech, Inc. Sephadex and Schleicher and Schuell microporous layers and trifluoroacetic acid were purchased from Vinnells Chemical Co.

Modification of Glutamate Dehydrogenase with [15-S]-PSBA - Glutamate dehydrogenase, purchased from Schlesinger and Schuscll microporous layers and trifluoroacetic acid were purchased from Vinnells Chemical Co. Dehydrogenase, 50 mg, was dissolved in 0.1 M potassium phosphate buffer, pH 7.1, and dialyzed overnight at 4°C. The dialyzed material was centrifuged to remove the insoluble material and denatured protein. The concentration of protein was determined from the absorbance at 280 nm using an ε = 1.55 × 10^4 cm^-1 mol^-1 or 1.85 × 10^4 cm^-1 mol^-1 (12).

Thermolyse, thioglycolic acid, and hydrolysis were carried out by Sigma Chemical Co. Phenylboronate agarose, Mono Q 5/5, was from Amersham and Pharmacia Biotech, Inc. Sephadex and Schleicher and Schuell microporous layers and trifluoroacetic acid were purchased from Vinnells Chemical Co.

Modification of Glutamate Dehydrogenase with [15-S]-PSBA - Glutamate dehydrogenase, purchased from Schlesinger and Schuscll microporous layers and trifluoroacetic acid were purchased from Vinnells Chemical Co. Dehydrogenase, 50 mg, was dissolved in 0.1 M potassium phosphate buffer, pH 7.1, and dialyzed overnight at 4°C. The dialyzed material was centrifuged to remove the insoluble material and denatured protein. The concentration of protein was determined from the absorbance at 280 nm using an ε = 1.55 × 10^4 cm^-1 mol^-1 or 1.85 × 10^4 cm^-1 mol^-1 (12).

Thermolyse, thioglycolic acid, and hydrolysis were carried out by Sigma Chemical Co. Phenylboronate agarose, Mono Q 5/5, was from Amersham and Pharmacia Biotech, Inc. Sephadex and Schleicher and Schuell microporous layers and trifluoroacetic acid were purchased from Vinnells Chemical Co.

Modification of Glutamate Dehydrogenase with [15-S]-PSBA - Glutamate dehydrogenase, purchased from Schlesinger and Schuscll microporous layers and trifluoroacetic acid were purchased from Vinnells Chemical Co. Dehydrogenase, 50 mg, was dissolved in 0.1 M potassium phosphate buffer, pH 7.1, and dialyzed overnight at 4°C. The dialyzed material was centrifuged to remove the insoluble material and denatured protein. The concentration of protein was determined from the absorbance at 280 nm using an ε = 1.55 × 10^4 cm^-1 mol^-1 or 1.85 × 10^4 cm^-1 mol^-1 (12).

Thermolyse, thioglycolic acid, and hydrolysis were carried out by Sigma Chemical Co. Phenylboronate agarose, Mono Q 5/5, was from Amersham and Pharmacia Biotech, Inc. Sephadex and Schleicher and Schuell microporous layers and trifluoroacetic acid were purchased from Vinnells Chemical Co.

Modification of Glutamate Dehydrogenase with [15-S]-PSBA - Glutamate dehydrogenase, purchased from Schlesinger and Schuscll microporous layers and trifluoroacetic acid were purchased from Vinnells Chemical Co. Dehydrogenase, 50 mg, was dissolved in 0.1 M potassium phosphate buffer, pH 7.1, and dialyzed overnight at 4°C. The dialyzed material was centrifuged to remove the insoluble material and denatured protein. The concentration of protein was determined from the absorbance at 280 nm using an ε = 1.55 × 10^4 cm^-1 mol^-1 or 1.85 × 10^4 cm^-1 mol^-1 (12).

Thermolyse, thioglycolic acid, and hydrolysis were carried out by Sigma Chemical Co. Phenylboronate agarose, Mono Q 5/5, was from Amersham and Pharmacia Biotech, Inc. Sephadex and Schleicher and Schuell microporous layers and trifluoroacetic acid were purchased from Vinnells Chemical Co.

Modification of Glutamate Dehydrogenase with [15-S]-PSBA - Glutamate dehydrogenase, purchased from Schlesinger and Schuscll microporous layers and trifluoroacetic acid were purchased from Vinnells Chemical Co. Dehydrogenase, 50 mg, was dissolved in 0.1 M potassium phosphate buffer, pH 7.1, and dialyzed overnight at 4°C. The dialyzed material was centrifuged to remove the insoluble material and denatured protein. The concentration of protein was determined from the absorbance at 280 nm using an ε = 1.55 × 10^4 cm^-1 mol^-1 or 1.85 × 10^4 cm^-1 mol^-1 (12).

Thermolyse, thioglycolic acid, and hydrolysis were carried out by Sigma Chemical Co. Phenylboronate agarose, Mono Q 5/5, was from Amersham and Pharmacia Biotech, Inc. Sephadex and Schleicher and Schuell microporous layers and trifluoroacetic acid were purchased from Vinnells Chemical Co.

Modification of Glutamate Dehydrogenase with [15-S]-PSBA - Glutamate dehydrogenase, purchased from Schlesinger and Schuscll microporous layers and trifluoroacetic acid were purchased from Vinnells Chemical Co. Dehydrogenase, 50 mg, was dissolved in 0.1 M potassium phosphate buffer, pH 7.1, and dialyzed overnight at 4°C. The dialyzed material was centrifuged to remove the insoluble material and denatured protein. The concentration of protein was determined from the absorbance at 280 nm using an ε = 1.55 × 10^4 cm^-1 mol^-1 or 1.85 × 10^4 cm^-1 mol^-1 (12).

Thermolyse, thioglycolic acid, and hydrolysis were carried out by Sigma Chemical Co. Phenylboronate agarose, Mono Q 5/5, was from Amersham and Pharmacia Biotech, Inc. Sephadex and Schleicher and Schuell microporous layers and trifluoroacetic acid were purchased from Vinnells Chemical Co.