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Bovine Liver Glutamate Dehydrogenase: Tentative Amino Acid Sequence; Identification of a Reactive Lysine; Nitration of a Specific Tyrosine and Loss of Allosteric Inhibition by Guanosine Triphosphate*

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Abstract. A tentative but almost complete amino acid sequence for the subunit peptide chain of bovine liver glutamate dehydrogenase indicates a minimal size of 506 residues with a molecular weight of 56,100, in accord with the physical size of the subunit of 55,900. Inactivation with pyridoxal 5′-phosphate, followed by reduction with sodium borohydride, has permitted identification of the essential lysine as residue 97. Nitration of tyrosine-412 is accompanied by loss of the allosteric inhibitory effect of guanosine triphosphate.

Comparison of the sequences of glutamate dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase has indicated that only two 12-residue sequences are similar in the two enzymes; this sequence includes reactive lysine-97 of the former enzyme.

Glutamate dehydrogenase (EC 1.4.1.3) occupies a central position in mammalian nitrogen metabolism since the reaction which it catalyzes provides the major pathway for the interconversion of α-amino group nitrogen and ammonia. Other important enzymes of amino acid metabolism usually employ pyridoxal 5′-phosphate in transamination or decarboxylation as the initial reaction step. The dehydrogenase is also of interest because its activity is subject to allosteric regulation by a variety of nucleoside polyphosphates, e.g., GTP is a strong inhibitory effector and ADP an activator.[1] We have undertaken a study of the structure of glutamate dehydrogenase[2] for a variety of reasons: first, because little is yet known regarding the structures and active sites of dehydrogenases; second, because this enzyme could serve as a useful model in attempts to understand the regulatory processes of multichain enzymes in general and of dehydrogenases in particular; third, because it would be useful to ascertain possible evolutionary relationships among the many dehydrogenases that utilize the pyridine nucleotide coenzymes, DPN and TPN. At present, only the sequence of glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) is completely known,[3] but work is also in progress on the alcohol dehydrogenases,[4] and an x-ray crystallographic study of lactate dehydrogenase (EC 1.1.1.27) has been reported.[5]

Although our work on the amino acid sequence of glutamate dehydrogenase is still not entirely complete, the major structural features of the enzyme are now clear. Furthermore, we have identified the location of the ϵ-amino group of a lysyl residue which is essential for activity,[6] and of a tyrosyl residue which upon nitration is accompanied by loss of the effect of GTP as an allosteric inhibitor.[7] In this preliminary communication we shall report briefly on these results. Full details of these studies will be reported elsewhere.

Amino Acid Sequence. Bovine liver glutamate dehydrogenase in its active minimal size of approximately 313,000 mol wt consists of six subunit polypeptide chains.[8] We have found that the molecular weight of the individual chains, as determined by sedimentation-equilibrium measurements in guanidine hydrochloride under reducing conditions, is approximately 55,900, in reasonable accord with earlier reports.[8,9]

Our sequence studies indicate that the six polypeptide chains are identical and that each contains 506 amino acid residues, the calculated molecular weight being 56,100. Our presently derived tentative sequence is shown in Figs. 1–3.

The peptides shown in the figures were obtained as follows: from the *S*-carboxymethylated protein by tryptic hydrolysis (T),[10,11] by peptic hydrolysis (P),[12] by cleavage with cyanogen bromide (CNBr),[13] and from a mixture of two large CNBr peptides by hydrolysis with trypsin and thermolysin (L)[14]; also, from tryptic hydrolysis of the carboxymethylated and maleylated protein (TM)[15] and from a tryptic digest of the oxidized and maleylated protein (TOM).[15] Detailed sequence studies were performed mainly on tryptic peptides[16]; other peptides were used to supplement this sequence information, to obtain overlapping peptides, and to confirm many individual portions of the sequence. The figures include only those peptides which contributed in major fashion to the derivation of the sequence.

In particular, we have shown only the larger or overlapping peptic peptides, those peptides, TM and TOM, which were not isolated from the tryptic digest or which contributed overlaps, and only a few of the thermolysin (L) peptides.

At present, we have established the NH_2-terminal sequence of 50 residues placed by virtue of the sequence Ala-Asp- shown to be at the NH_2-terminus of the intact subunit chain by Edman degradation,[11] the COOH-terminal sequence of 137 residues placed by knowledge of the carboxyl terminus of the intact subunit chain, previously shown to be -Phe-ThrCOOH,[17] and an interior segment comprising 319 residues. The two gaps between these three segments are shown by two vertical bars. Thus, two additional overlapping peptides are required to complete the sequence. Inasmuch as all of the peptides that have been isolated to date fit the present sequence, there is no evidence suggesting that any residues are missing in the two gaps. Furthermore, both the amino acid composition and the calculated molecular weight that have been derived from the sequence are in good agreement with the experimentally determined values. In addition to the uncertainty indicated by the two gaps, the possible amide character of some residues is unknown and the exact sequences of a few residues, indicated by parentheses, remain to be elucidated. For convenience, we shall use the tentative numbering of the residues indicated in the figures.

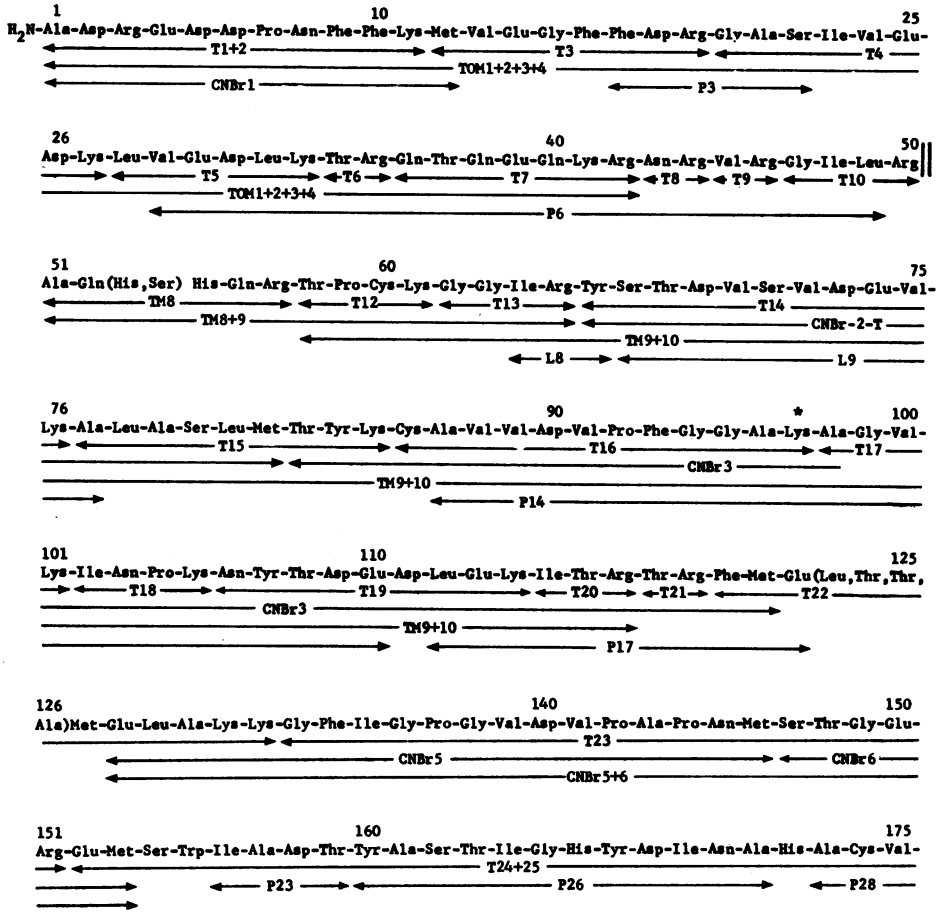

FIG. 1. The amino acid sequence and alignment of peptides derived from bovine liver glutamate dehydrogenase, tentatively residues 1 through 175. The lysyl residue found to react with pyridoxal 5'-phosphate[6], residue 97, is indicated by an asterisk. No overlapping peptide is available as yet for linking residues 50 and 51.

Certain aspects of the sequence work merit comment either because of unusual difficulties or because of special features of the sequence. One interesting property of the molecule is that two Asp-Pro bonds were generally found to be almost completely hydrolyzed in the tryptic, peptic, and CNBr digests[18]; the labile bonds were between residues 6 and 7 and between residues 264 and 265 (see Figs. 1 and 2). Inasmuch as the peptic digest was performed at pH 2.2 and the other digests were exposed to acidic pH values during chromatographic separation of the peptides, or during the cleavage with CNBr, and it is known that aspartyl peptides can be selectively hydrolyzed under acidic conditions, the anomalous scission of the Asp-Pro bonds may be attributed to hydrolysis by intramolecular β-carboxyl group catalysis. The unusual lability of the Asp-Pro bonds must be a consequence of the properties of both residues.

Peptide CNBr-11 was readily isolated by countercurrent distribution and,

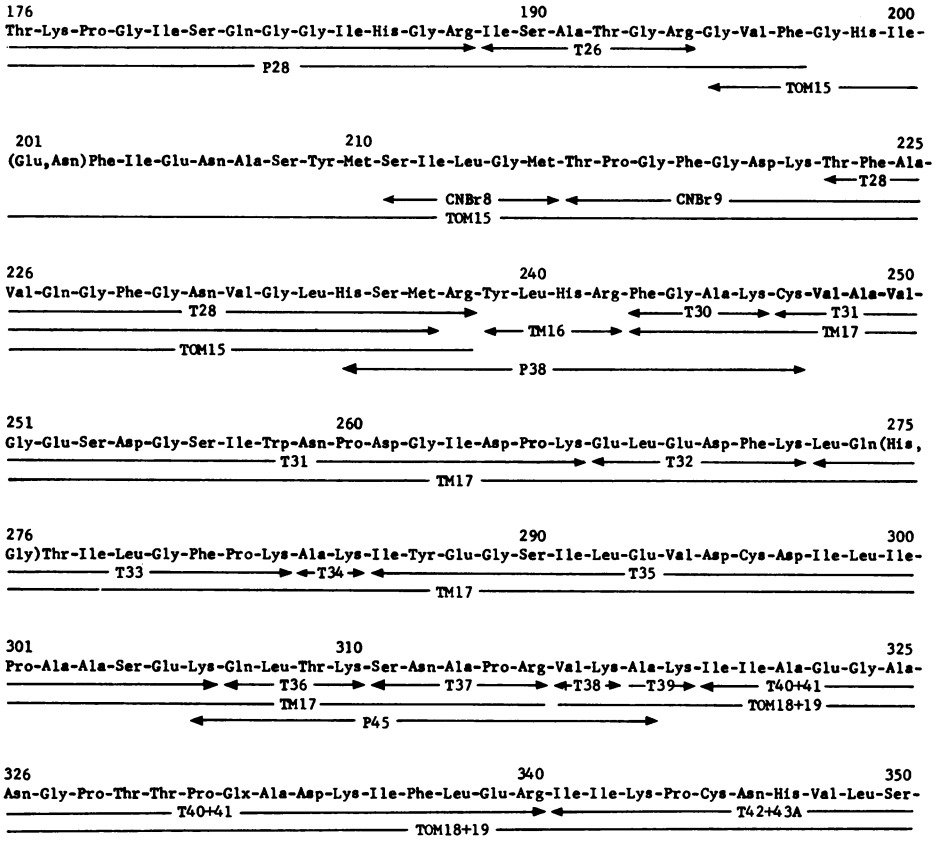

FIG. 2. The amino acid sequence and alignment of peptides derived from bovine liver glutamate dehydrogenase, tentatively residues 176 through 350.

in fact, was easily extracted from acidic solution into 2-butanol.[14] This peptide is rich in hydrophobic residues and proved to be sparingly soluble in aqueous solution and only slowly hydrolyzed by proteolytic enzymes. The COOH-terminal portion of the sequence, comprising residues 371 through 506, is rich in hydrophobic residues and contains eight of the 18 tyrosyl residues of the protein. In contrast, the NH_2-terminal portion of the molecule proved to be rich in ionic residues, particularly arginine; indeed, the first 50 residues include no tyrosine and 22 ionic residues.

Cleavage with CNBr was incomplete. As already noted,[19] Met-Ser and Met-Thr bonds are not always cleaved during the conversion of methionine to homoserine by this reagent. Although this permitted the isolation of a few joined peptides (CNBr-5+6, CNBr-11+12, and CNBr-13+14), it did, however, preclude the isolation in pure form of some of the larger peptides because of the presence of many incompletely fragmented portions of the molecule. As yet, efforts by a variety of methods to isolate in pure form CNBr-2 (residues 13 through 82) and CNBr-10 (residues 238 through 372) have been unsuccessful,

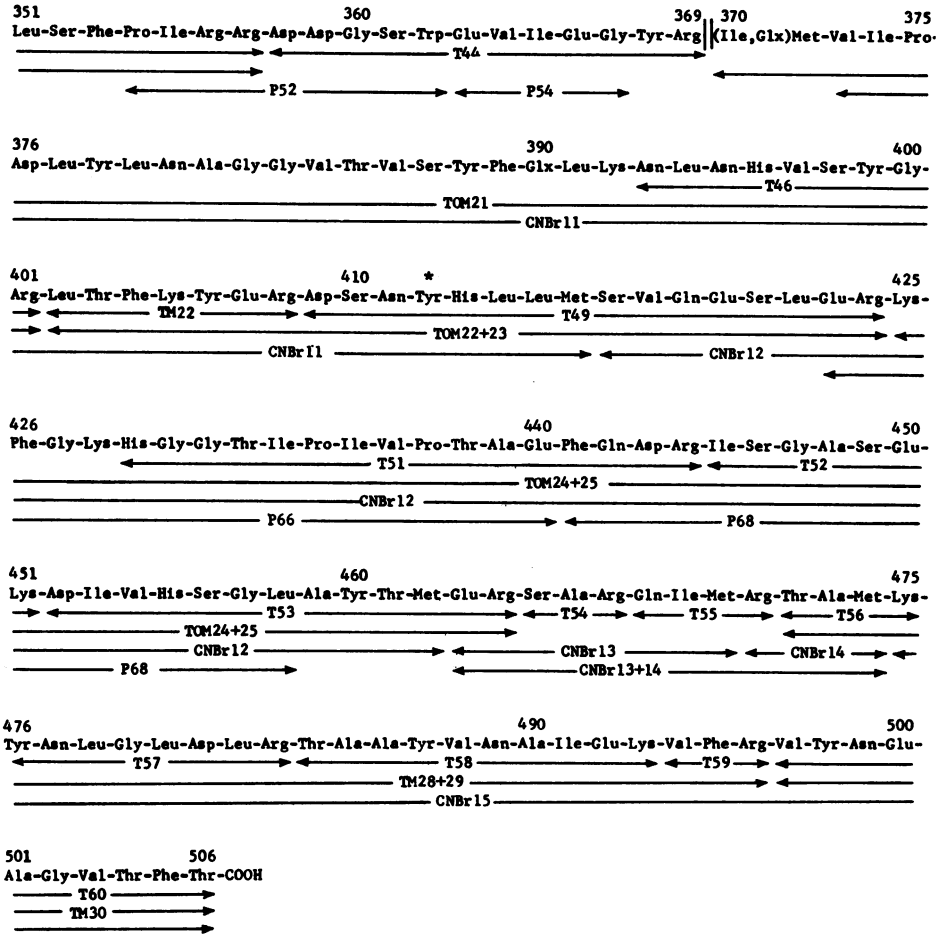

FIG. 3. The amino acid sequence and alignment of peptides derived from bovine liver glutamate dehydrogenase, tentatively residues 351 through 506. The tyrosyl residue found[7] to be rapidly nitrated by tetranitromethane with simultaneous loss of GTP inhibitory effect, residue 412, is indicated by an asterisk. No peptide is available as yet for linking residues 369 and 370.

These peptides, which span the two gaps in the present sequence, would aid in completing the sequence.

Of particular value in obtaining large, overlapping peptides was the use of maleylated protein that had previously been oxidized with performic acid. This material was rapidly but incompletely hydrolyzed with trypsin and yielded many peptides which contained not only one or more lysyl residues, as expected,[20] but also included as many as three arginyl residues in addition to the one always expected at the COOH-terminus of each peptide.

Reactive Lysyl Residue. Pyridoxal 5'-phosphate inhibits GDH.[21] Reduction of the pyridoxal 5'-phosphate-inactivated enzyme with $NaBH_4$, followed by alkylation of the thiol groups with iodoacetate and then by tryptic hydrolysis, yielded a single peptide having the fluorescence characteristic of ϵ-pyridoxyl

residues. After purification and hydrolysis the peptide contained one equivalent of ε-pyridoxyl lysine. The sequence[6] of the fluorescent peptide (tentatively, residues 86 through 101) is:[22] Cys-Ala-Val-Val-Asp-Val-Pro-Phe-Gly-Gly-Ala-Lys*-Ala-Gly-Val-Lys. Clearly, the reactive lysyl residue is at position 97.

Nitration of Tyrosine and Effect of GTP. Price and Radda have reported[23] that treatment of the dehydrogenase with tetranitromethane abolishes the sensitivity of the enzyme to the allosteric inhibitor GTP, with simultaneous nitration of a single tyrosyl residue. We have confirmed this observation and have found[7] that the rate of formation of 3-nitrotyrosine during the reaction of glutamate dehydrogenase with tetranitromethane is apparently the sum of two reactions: an initial rapid reaction which accounts for the nitration of approximately one tyrosyl residue per subunit chain, and a slower reaction which also accounts for significant tyrosine nitration. Reaction of the enzyme with tetranitromethane, followed by alkylation of the thiol groups and tryptic digestion, resulted in the isolation of a single peptide containing 3-nitrotyrosine.[7] This peptide (T-49; Fig. 3) had the sequence: Asp-Ser-Asn-Tyr*-His-Leu-Leu-Met-Ser-Val-Gln-Glu-Ser-Leu-Glu-Arg (tentatively residues 409 through 424).

This result was also confirmed by the independent isolation of CNBr-11 and CNB-11+12 (Fig. 3) and identification of the same residue as 3-nitrotyrosine. The modified tyrosine, residue 412, is the 94th residue from the COOH-terminus and is remote from lysine-97 which is reactive with pyridoxal 5′-phosphate.

The reaction with tetranitromethane results in a simultaneous low level of modification of other tyrosyl residues and a small amount of cysteine oxidation, but no alteration of other residues in the protein. Inasmuch as others[23,24] have used different reagents for modifying tyrosine with concomitant abolition of the allosteric inhibition by GTP, it appears that nitration of tyrosine-413 is responsible for this effect.

Relationship of Glutamate Dehydrogenase to Other Enzymes. Comparison of the tentative sequence for glutamate dehydrogenase with the sequence for porcine glyceraldehyde-3-phosphate dehydrogenase (GPDH),[3] both by visual inspection and by a Fortran computer program designed to detect segments of identical or of genetically similar (single base changes) sequences, has revealed that only the region containing the sequence with the reactive lysyl residue of glutamate dehydrogenase appears to possess any significant relationship to the sequence of GPDH (Fig. 4). In these twelve residue sequences, six of the positions are occupied by identical residues, whereas the others are occupied by residues related by a single base change within their respective triplet nucleotide codons. If the two enzymes had been derived from a common, remote, ancestral gene by duplication and independent evolution, only the the sequence including the active site lysine of the glutamate enzyme has been retained in re-

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        94                                  105
GDH     GLY-GLY-ALA-LYS-ALA-GLY-VAL-LYS-ILE-ASN-PRO-LYS

GPDH    GLY-ALA-ALA-LYS-ALA-VAL-GLY-LYS-VAL-ILE-PRO-GLU
        209                                 220
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FIG. 4. Homologous sequences of bovine liver glutamate dehydrogenase (GDH) and porcine glyceraldehyde-3-phosphate dehydrogenase (GPDH)[3]

cognizable degree. It should be noted that GPDH contains a reactive lysine at position 183 in a sequence which is not homologous with that surrounding the reactive lysine of glutamate dehydrogenase. Whether the counterpart of reactive lysine 97 in glutamate dehydrogenase—that is, residue-212 in GPDH—has any special function or reactivity in the latter enzyme is at present unknown. The presently available partial sequences of the two alcohol dehydrogenases[4] show no evident relationship to glutamate dehydrogenase.

Abbreviations: GDH, glutamate dehydrogenase; GPDH, glyceraldehyde-3-phosphate dehydrogenase; T, from the tryptic digest; P, from the peptic digest; CNBr, from cleavage with cyanogen bromide; L, from a thermolysin digest of large CNBr peptides; TM, from the tryptic digest of the maleylated, carboxymethylated protein; TOM, from the tryptic digest of maleylated oxidized protein.

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