Bovine Liver Glutamate Dehydrogenase

SEQUENCE OF A HEXADECAPEPTIDE CONTAINING A LYSYL RESIDUE REACTIVE WITH PYRIDOXAL 5'-PHOSPHATE*

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DENNIS PISZKIEWICZ, MICHAEL LANDON, AND EMIL L. SMITH†
From the Department of Biological Chemistry, UCLA School of Medicine, Los Angeles, California 90024

SUMMARY

Glutamate dehydrogenase is reversibly inhibited by the reaction of 1 mole of pyridoxal 5'-phosphate per mole of subunit polypeptide chain of the enzyme. Reduction of the pyridoxal 5'-phosphate-inactivated enzyme with NaBH₄, followed by alkylation of the thiol groups and subsequent tryptic digestion, yielded a single fluorescent peptide which after acid hydrolysis, gave approximately 1 eq of ε-pyridoxyllysine. This peptide was recognized by its amino acid composition as an overlap of two tryptic peptides which had been isolated and sequenced independently. The hexadecapeptide has the sequence: Cys-Ala-Val-Val-Anp-Pro-Val-Phe-Gly-Gly-Ala-Lys*-Ala-Gly-Val-Lys.

For several years our laboratory has been engaged in a study of the amino acid sequence of bovine liver glutamate dehydrogenase (L-glutamate:DPN (TPN) \(^1\) oxidoreductase (deaminating), EC 1.4.1.3). We have found that the six subunit polypeptide chains are identical and contain a minimum of 507 residues. A tentative sequence has been almost completely deduced from peptides derived from tryptic, cyanogen bromide, and peptic digests of the S-carboxymethylated protein and from the tryptic digests of the maleylated S-carboxymethylated and the maleylated oxidized protein. Having elucidated most of the sequence of the enzyme, we have now begun to investigate the functional groups which may be involved in its catalytic and regulatory behavior.

Anderson, Anderson, and Churchich (1) have reported that the enzyme is inactivated by incubation with pyridoxal 5'-P. This inactivation was reversed by dialysis and stabilized by NaBH₄ reduction. ε-Pyridoxyllysine was identified in acid hydrolysates of the reduced phosphorylpyridoxyl-enzyme. By assuming a molecular weight of 56,000 per polypeptide chain of the enzyme is inactivated by incubation with pyridoxal P. This inactivation was reversed by dialysis and stabilized by NaBH₄ reduction. ε-Pyridoxyllysine was identified in acid hydrolysates of the reduced phosphorylpyridoxyl-enzyme. By assuming a molecular weight of 56,000 per polypeptide chain of the enzyme, we have now begun to investigate the functional groups which may be involved in its catalytic and regulatory behavior.

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The course of the reaction of pyridoxal-5'-P (3.2 x 10^{-4} M) with the dehydrogenase (4 x 10^{-5} M) at 40° in 0.05 M phosphate buffer at pH 7.30 is shown in Fig. 1. Aliquots of the reaction mixture were added to assay mixtures, and the initial velocities of DPN reduction were determined at 25° (1). When pyridoxal-5'-P was added to the enzyme in a molar ratio of 80:1, activity dropped rapidly to a minimum after about 15 min. Thus, the observation of glutamate dehydrogenase inactivation by pyridoxal-5'-P (1) was confirmed.

Isolation and Characterization of Pyridoxal-5'-P-labeled Peptide—The dehydrogenase (10 μmoles), which was inactivated with pyridoxal-5'-P, was reduced with NaBH₄. It was then carboxymethylated and hydrolyzed with trypsin, as described above. The tryptic digest was applied to a column of Dowex 1-X2. The elution profile of the tryptic peptides is shown in Fig. 2. Aliquots of all pooled fractions were run on descending paper chromatography. Only Fraction 1 showed the fluorescence characteristic of a pyridoxal-5'-P-derivative.

Fraction 1 from the Dowex 1-X2 column was then applied to a column of Sephadex G-50; its elution profile is shown in Fig. 3. Fractions A and B, which contained fluorescent material, were concentrated by rotary evaporation. Each fraction was then applied to paper for descending chromatography. Although six or seven ninhydrin-positive bands could be detected, only one band (RF, 0.22) was fluorescent under ultraviolet light. This peptide was eluted from paper with 30% aqueous acetic acid.

The acidic hydrolysate of the pyridoxal-5'-P-labeled peptide...
reactive lysine of glutamate dehydrogenase

Peptide T1—The composition of Peptide T1 was ascertained by acidic hydrolysis for 24 and 72 hours and by hydrolysis with aminopeptidase M (9). The results indicate the presence of 1 residue each of Cm-cysteine and aspartic acid; asparagine was absent from the enzymic digest. All of the data for this peptide are summarized in Table I.

Five steps of the Edman degradation allowed identification of the NH₄-terminal sequence as Cm-Cys-Ala-Val-Asp. Hydrolysis by carboxypeptidases A plus B released lysine and almost as much alanine, indicating the COOH-terminal sequence as Ala-Lys. Significant quantities of glycine and phenylalanine were also liberated, but the presence of 2 glycine residues in the peptide allowed several possible interpretations of these data.

Hydrolysis with chymotrypsin yielded two peptides which were separated by preparative paper electrophoresis at pH 1.9. Peptide T1-C1 had a lower electrophoretic mobility than Peptide T1-C2. The composition of Peptide T1-C1 indicated that it was derived from the NH₄ terminus of the parent peptide. Since the NH₄-terminal pentapeptide sequence of Peptide T1 had been determined, the COOH terminus of Peptide T1-C1 was obviously (Pro,Val)-Phe, in accord with chymotryptic hydrolysis at the aromatic residue. Carboxypeptidase A, which does not remove residues adjacent to prolyl residues, liberated only phenylalanine from Peptide T1-C1, indicating the COOH-terminal sequence to be Pro-Val-Phe. Peptide T1-C2 contained alanine and lysine, which were COOH-terminal in the parent peptide, plus 2 residues of glycine which must be NH₄-terminal. Two steps of the Edman degradation confirmed the sequence Gly-Gly for the NH₄ terminus, and indicated the sequence of Peptide T1-C2 to be Gly-Gly-Ala-Lys.

Hydrolysis of the peptide with carboxypeptidase B for 5 hours released only lysine, whereas hydrolysis with carboxypeptidases A pH 3.5 released alanine, glycine, and lysine in apparently equivalent amounts (paper electrophoresis at pH 1.9). Thus, the COOH-terminal terminal sequence is (Ala,Gly,Val)-Lys.

The above results indicate that the labeled component has the composition of a hexadecapeptide containing 1 residue of unlabeled lysine at the COOH terminus and 1 residue of pyridoxal-5′-P-lysine substituted at the ϵ-amino group. Thus the isolated material should represent the sum of the compositions of two overlapping tryptic peptides. Comparison of the composition of the pyridoxal-5′-P-peptide with the sequence data obtained for unlabeled glutamate dehydrogenase revealed that the sum of two tryptic peptides uniquely fitted the composition of the hexadecapeptide. Further, the COOH-terminal data obtained with the labeled peptide were consistent with the composition of a unique tetrapeptide derived from the protein (see below, Peptide T2). The isolation of these two tryptic peptides will be presented together with others isolated from the same digest,1 but the sequence information is given here in order to render complete the description of the pyridoxal-5′-P-hexadecapeptide. For convenience the complete sequence is shown in Fig. 4 with the two tryptic peptides and an overlapping peptide derived from a peptic digest.2

1 The abbreviations used are: Cm, carboxymethyl; PTH, phenylthiohydantoin.
2 The values for Cm-cysteine and ϵ-pyridoxyllysine are not corrected for destruction during acid hydrolysis.
3 Hydrolysis for 72 hours.

*Fig. 4. Amino acid sequence of the pyridoxal-5′-P-hexadecapeptide as derived by studies of two tryptic peptides (T1 and T2) obtained from the dehydrogenase and from a peptide (Pe) obtained from a peptic digest. SCMC, S-carboxymethylcysteine; PLP, pyridoxal-5′-P.*

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1. *Hydrolysis for 72 hours.*
2. *The abbreviations used are: Cm, carboxymethyl; PTH, phenylthiohydantoin.*
3. *The values for Cm-cysteine and ϵ-pyridoxyllysine are uncorrected for destruction during acid hydrolysis.*
DISCUSSION

The results reported above show that pyridoxal 5'-phosphate inactivates glutamate dehydrogenase by covalently bonding to the ε-amino group of a specific lysine in the protein. After NaDH reduction and tryptic digestion, a hexadecapeptide containing this pyridoxal-5'-P-labeled lysine residue was isolated. Its composition uniquely places it as an overlap of two tryptic peptides which were isolated independently. Examination of the sequence information presently available for the polypeptide chain of the dehydrogenase indicates that this hexadecapeptide comprises residues 86 to 101 and places the labeled lysyl residue around this labeled lysine, as presented by these authors, in comparison with that of the pyridoxal-5'-P-labeled hexadecapeptide determined in this investigation and that given by Holbrook and Jeckel for a peptide labeled with a maleimide derivative (ASPM). SCMC, S-carboxymethylcysteine; PLP, pyridoxal-5'-P.

which could accommodate the partial sequence deduced by them. Thus, we conclude that pyridoxal phosphate and the substituted maleimide react with the same lysyl residue of the dehydrogenase.

The differences between the two sequences (Fig. 5) can probably be attributed in part to an incorrect amino acid analysis for the maleimide substituted peptide. Thus, proline, which gives a low color yield with ninhydrin (6), was presumably over-looked or considered to be negligible in quantity. Also, cysteine was not detected in their study, but an extra residue of aspartic acid was reported in their analysis (11). It is conceivable that during reaction with the maleimide or in the subsequent handling cysteine was oxidized (or modified in another manner) to yield a product chromatographically similar to aspartic acid was reported in their analysis (11). It is conceivable that during reaction with the maleimide or in the subsequent handling cysteine was oxidized (or modified in another manner) to yield a product chromatographically similar to aspartic acid.

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Fig. 5. Comparison of the sequences of the pyridoxal-5'-P-hexadecapeptide determined in this investigation and that given by Holbrook and Jeckel (11) for a peptide labeled with a maleimide derivative (ASPM). SCMC, S-carboxymethylcysteine; PLP, pyridoxal-5'-P.

6 Note Added in Proof—An earlier tentative sequence alignment (10) had indicated that the active lysine is residue 151; this is clearly incorrect on the basis of additional overlapping peptides recently isolated. Although a peptide is still required to overlap a gap between residues 50 and 51, the present assignment appears to be on a firmer basis.
a lysyl residue. Colman and Frieden (12) have shown that acetic anhydride at low concentration reacts with one amino group per subunit chain to cause inactivation. Similarly, 2,4,6-trinitrobenzenesulfonic acid (13) and carbamyl phosphate (14) inactivate the enzyme by reacting with approximately one lysine ε-amino group per subunit chain. In view of the specific reactions of pyridoxal-5'-P and N-(N'-acetyl-4-sulfamoylphenyl)maleimide with the enzyme, one may reasonably expect all of these reagents to inactivate glutamate dehydrogenase by covalently bonding to the ε-amino group of the lysyl residue tentatively assigned to position 97.

It should be recalled that glutamate dehydrogenase is one of the few enzymes involved in amino acid metabolism that does not require pyridoxal-5'-P as a cofactor. The participation of pyridoxal-5'-P-dependent enzymes in other pathways for the metabolism of glutamic acid suggests that the reaction of pyridoxal-5'-P with the dehydrogenase may function physiologically as a regulatory effector in amino acid metabolism. Before the nature and significance of this inhibitory reaction can be fully appraised, however, a thorough study is required of the effect of this substance in relation to substrates and other effectors. Such an investigation is now in progress.

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REFERENCES
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