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Active-Center Peptides of Liver-Alcohol Dehydrogenase. I. The Sequence Surrounding the Active Cysteinyl Residues*

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Previous work has demonstrated that the two zinc atoms and two of its twenty-eight sulfhydryl groups are essential to the catalytic activity of horse liver–alcohol dehydrogenase. The two essential —SH groups were carboxymethylated with [1-^{14}C]iodoacetate and the enzyme was digested with trypsin. Comparison of the resultant number of peptides with the number of trypsin-susceptible bonds suggests that horse liver–alcohol dehydrogenase consists of two similar or identical chains. The isolation of a single [^{14}C]carboxymethylcysteinyloctapeptide by gel filtration and ion-exchange chromatography indicates that the cysteinyl residues are part of an identical sequence in these two chains. The sequence of the peptide is Met-Val-Ala-Thr-Gly-Ileu-*S*-carboxymethylcysteine-Arg. This peptide does not bind the functional zinc atoms of the enzyme.

We have previously emphasized that the characteristics of metalloenzymes allow for experimental approaches which, in combination with other existent procedures, offer advantages in the elucidation of the active centers of such enzymes (Vallee, 1961). Our long-standing interest in the mechanism of action of the zinc-containing, DPN-dependent, horse liver–alcohol dehydrogenase has now led to an investigation of the composition and structure of its active center by means of site-specific and selective reagents. The loss of enzymatic activity due to the modification of side chains of amino acids has long served as a valuable means for the identification of residues which are indispensable in the mechanism of action. This mode of procedure has served further as a guide to the isolation of the peptide sequence surrounding such "active residues."

We have reported briefly (Li and Vallee, 1963) that iodoacetate preferentially carboxymethylates 2 of the 28 thiol groups of liver-alcohol dehydrogenase, resulting in simultaneous loss of activity. The present communication describes the isolation and characterization of the peptides containing these carboxymethylcysteinyl residues.

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MATERIALS AND METHODS

Crystalline alcohol dehydrogenase of horse liver was obtained from C. F. Boehringer und Soehne, Mannheim, W. Germany. Before use, the enzyme was dialyzed for 5 days against 0.1 M sodium phosphate buffer, *p*H 7.5, 4°, to remove low-molecular-weight impurities which absorb radiation at 280 mμ. The concentration of protein was determined by measurement of the absorbance at 280 mμ, based upon an absorptivity of 0.455 mg^{-1} cm^2 (Bonnichsen, 1950). Molar concentrations are based on a molecular weight of 83,300 (Ehrenberg and Dalziel, 1958). The turnover number of the enzyme preparations varied from 480 to 530 moles DPN/min per mole protein, and the —SH titer (Boyer, 1954) of such preparations varied between 26 and 28 moles —SH/mole protein.

Succinyltrypsin was prepared from twice-crystallized trypsin (Worthington Biochemical Corp.) ac-

cording to Terminiello *et al.* (1958) and stored at 4° as a lyophilized powder.

[1-^{14}C] Iodoacetate (2 mc/mmole) was obtained from New England Nuclear Corp., diluted 1:8 (w/w) with unlabeled iodoacetate (Matheson, Coleman and Bell), and dissolved in 0.1 M phosphate. The final *p*H of the solution was adjusted to 7.5 with dilute NaOH. All reagents were reagent grade or highest purity grade chemicals.

Carboxymethylation and Digestion of Liver-Alcohol Dehydrogenase.—Liver-alcohol dehydrogenase (200 mg) was exposed to a 600-fold molar excess of [^{14}C] iodoacetate in 24 ml of 0.1 M phosphate buffer, *p*H 7.5, 22°, for 165 minutes. The reaction was terminated by passing the solution over a 4.3 × 26-cm column of Sephadex G-25 (coarse beads; Pharmacia, Sweden). The labeled protein was separated from the excess reagent by elution with water which had been adjusted to *p*H 8–9 with ammonium hydroxide. The protein fractions were pooled and digested with succinyltrypsin at *p*H 8.0, 37°. The *p*H was maintained at 8.0 by the continuous addition of dilute ammonium hydroxide from a *p*H-stat (Radiometer) with constant stirring. During the 24 hours of digestion, three or four aliquots of succinyltrypsin were added successively to the reaction mixture up to a total of 8 mg. The digest was centrifuged to remove the small amount of precipitate formed, and the supernatant solution was either lyophilized or concentrated to dryness by rotatory evaporation under reduced pressure.

Electrophoresis and Chromatography.—Electrophoresis of the digest was carried out on Whatman No. 3 paper at *p*H 1.9 in 4% formic acid or at *p*H 6.5 in pyridine acetate with a potential difference of 35 v/cm for 90 or 120 minutes. For peptide mapping, a companion strip was sewn onto another piece of paper (Richmond and Hartley, 1959) and subjected to descending chromatography in the second dimension for 14 hours, using 1-butanol–acetic acid–water (4:1:5) as the solvent. The distribution of peptides was determined by staining with ninhydrin.

Nagarse Digestion.—Digestion of the [^{14}C] carboxymethylcysteinyl peptide with Nagarse (Nagase & Co., Japan) was carried out in 0.1 M pyridine acetate buffer, pH 7.5, 37°. The peptide was incubated with 1:50 (w/w) of Nagarse for 3 hours, streaked on paper, and subjected to electrophoresis (35 v/cm, 2 hr) at *p*H 1.9 in 4% formic acid. Marker strips were cut out, stained with ninhydrin, and scanned for ^{14}C activity to locate the resultant peptides, which were then eluted with 0.1 M pyridine acetate at *p*H 7.5.

Gel Filtration.—The dried sample was dissolved in 2 ml of water and applied to a 0.9 × 140-cm column of G-25 Sephadex (fine beads) equilibrated at 22° with 0.2% thiodiglycol either in water adjusted to *p*H 8 with ammonia or in 0.05 M ammonium carbonate buffer, *p*H 8. The digest was eluted with 0.2% thiodiglycol either in water or in 0.05 M ammonium carbonate, *p*H 8. Two-ml fractions were collected at a flow rate of approximately 10 ml/hr. The absorbance of the effluent fractions at 280 mμ was measured by means of a Zeiss spectrophotometer.

Ion-Exchange Chromatography.—Dowex 50-X2 resin (Bio-Rad, 200–325 mesh) was washed successively first with 2 N sodium hydroxide, then with water, and finally with 2 N hydrochloric acid. A 0.9 × 30-cm column of the resin was equilibrated with 0.2% thiodiglycol in 0.17 M pyridine acetate buffer, *p*H 4.7, 22°. The dried sample of peptide was dissolved in 1 ml of the buffer and applied to the column. Elution was initiated with 0.17 M pyridine acetate buffer, *p*H 4.7, followed by 0.4 M pyridine acetate, *p*H 4.6, 1.07 M pyridine acetate, *p*H 5.4, and 2.23 M pyridine acetate, *p*H 5.4 (Guidotti *et al.*, 1962); 0.2% thiodiglycol was added to all buffers in order to protect the carboxymethylcysteine label against oxidation. Two-ml fractions were collected at a flow rate of 20 ml/hr. The column was regenerated by washing with 500 ml of the starting buffer until the *p*H of the effluent was 4.7. The peptides in the effluent fractions were located by reaction with ninhydrin (Moore and Stein, 1948).

^{14}C Counting.—The number of moles of ^{14}C introduced into each mole of protein and peptide was determined on aliquots of the pooled fractions of the labeled protein and peptide by liquid scintillation counting (Packard, Model 3214). The position of ^{14}C activity on paper after electrophoresis and chromatography was detected by scanning the paper strips in an automatic chromatogram scanner (Vanguard Model 880). The ^{14}C activity of the effluent fractions from Sephadex and ion-exchange columns was measured by streaking 0.025-ml aliquots of each fraction along the width of a 3 × 90-cm strip of filter paper which was then read in the chromatogram scanner.

Amino Acid Analysis.—Quantitative amino acid analyses were performed with a Spinco Model 120B automatic amino acid analyzer according to the method of Spackman *et al.* (1958). The samples were hydrolyzed in sealed evacuated tubes with 6 N HCl at 105° for 24 and 72 hours, and prepared for analysis as described by Moore and Stein (1963). Before hydrolysis, pyridine acetate was removed by rotary evaporation under reduced pressure and the thiodiglycol was removed by extraction with ether. Performic acid oxidation was carried out as described by Moore (1963). Analysis for tryptophan was by the dimethylaminobenzaldehyde reaction (Spies and Chambers, 1948).

Sequential Peptide Degradation by Edman Procedure and Carboxypeptidase Digestion.—Reaction of phenylisothiocyanate with the peptide and cyclization to the phenylthiohydantoin (PTH)[1] derivative was performed as described by Margoliash (1962). The PTH amino acids were identified on paper using solvent systems I, II, and III of Sjoquist (1960). The amino acid composition of peptides after Edman degradation was determined on appropriate aliquots with the automatic amino acid analyzer or by paper electrophoresis. Digestion of the peptide by carboxypeptidase A and B (Worthington Biochemical Corp.) was carried out according to Guidotti *et al.* (1962).

Results

Incubation of liver-alcohol dehydrogenase with 600-fold molar excess of [^{14}C] iodoacetate in 0.1 M phosphate, *p*H 7.5 and 22° for 165 minutes completely inactivates the enzyme due to preferential carboxymethylation of 2 of its 28 cysteine residues (Li and Vallee, 1963).

The [^{14}C] carboxymethylated enzyme was digested with succinyltrypsin at *p*H 8.0, 37°, for 24 hours. The resultant peptides were separated on paper by two-dimensional electrophoresis and chromatography yielding a total of 36–38 peptide spots. Virtually all of the ^{14}C activity was localized in a single spot (Fig. 1).

On amino acid analysis, the ^{14}C-labeled peptide, eluted from paper, contained both arginine and lysine (Li and Drum, 1964), indicating heterogeneity of the material; carboxymethylcysteine could not be detected. Instead, a broad asymmetric peak eluting in the position of cysteic acid and two smaller peaks

[1] Abbreviations used in this work: PTH, phenylthiohydantoin; LADH, liver-alcohol dehydrogenase; SCM-cysteine, *S*-carboxymethylcysteine.

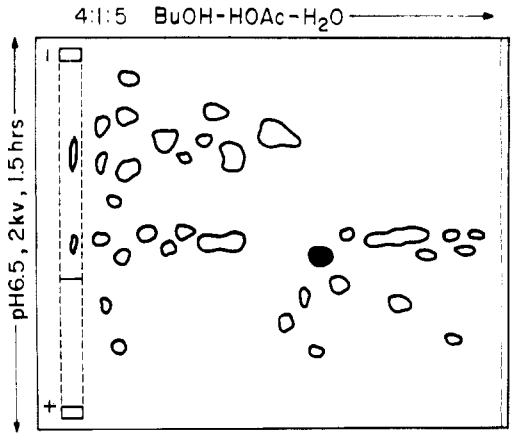

FIG. 1.—Peptide map of tryptic digest of [^{14}C]carboxymethyl-labeled liver-alcohol dehydrogenase. The tryptic digest was subjected to electrophoresis at *p*H 6.5 in pyridine acetate buffer for 90 minutes with a potential gradient of 35 v/cm, and then descended in the second dimension in butanol–acetic acid–water solvent. The peptide spots were identified by reaction with ninhydrin. Virtually all of the ^{14}C activity was localized in the black spot.

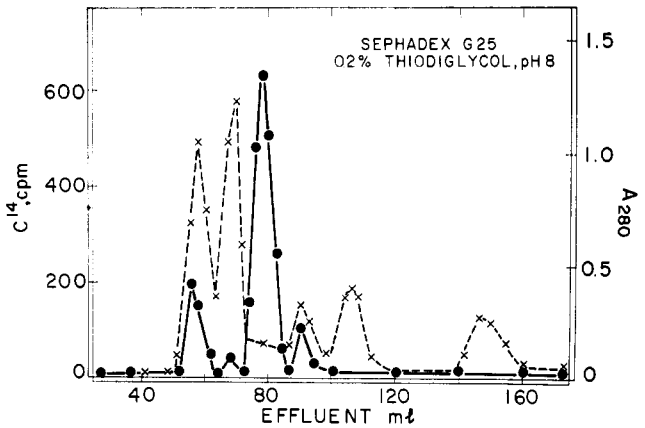

FIG. 2.—Gel filtration of tryptic digest of [^{14}C]carboxymethyl-labeled liver-alcohol dehydrogenase. The digest, dissolved in 2 ml of water, was applied to Sephadex G-25 (column, 0.9 × 140 cm) and eluted with 0.2% thiodiglycol in water, *p*H 8.0. Two-ml fractions were collected at a flow rate of 10 ml/hr. ^{14}C activity (●) and A_{280} (×) were measured as described under Methods. All excluded materials were eluted in the first 65 ml.

eluting a few ml thereafter were found, indicating decomposition of carboxymethylcysteine due to oxidation during paper chromatography.

Therefore, the tryptic digest was fractionated by gel filtration with Sephadex G-25 in the presence of 0.2% thiodiglycol to protect the [^{14}C] carboxymethylcysteinyl label against oxidation. Under these conditions, virtually all of the ^{14}C activity eluted as a single peak in the region of retarded low-molecular-weight peptides; only a small fraction of radioactivity emerged together with undigested or partially digested material (Fig. 2).

The ^{14}C-containing peptide fractions eluting between 76 and 82 ml (Fig. 2) were purified further by ion-exchange chromatography on Dowex 50-X2 (Fig. 3). Under these conditions, the major peak of ^{14}C activity emerged between 106 and 122 ml, coinciding exactly with one of the peaks of the ninhydrin-positive material (Fig. 3). The fractions under this peak were pooled. Since electrophoresis at *p*H 1.9 demonstrated only a single peptide, this material was hydrolyzed with 6 N HCl and analyzed for amino acids. One mole each of arginine, carboxymethylcysteine, threonine, glycine, alanine, valine, methionine, and isoleucine per mole of ^{14}C were found both at 24 and at 72 hours of hydrolysis. There were no decomposition products of carboxymethylcysteine nor was cysteic acid formed upon oxidation with performic acid. Tryptophan was not detected by means of the dimethylaminobenzaldehyde reaction (Spies and Chambers, 1948).

The sequence of this octapeptide was established by means of Edman degradation and digestion with Nagarse, and carboxypeptidase A and B. The following results were obtained:

(*a*) *Sequential Edman Degradation of the Octapeptide*

1st cycle: PTH methionine.

2nd cycle: PTH valine; residue: Arg 1.0, SCM-cysteine 0.8, Thr 0.8, Gly 1.1, Ala 1.0, Ileu, 0.9, *Val O, Met O.*

3rd cycle: PTH alanine; residue: Arg 1.0, SCM-cysteine 0.8, Thr 0.8, Gly 1.0, Ileu 1.1, *Ala trace*, Val O, Met O.

4th cycle: PTH threonine.

(*b*) *Carboxypeptidase Digestion of the Octapeptide.*—One mole of arginine per mole of peptide was liberated on incubation of the peptide with carboxypeptidase B for 1 hour. The next peptide bond is apparently not susceptible to hydrolysis by carboxypeptidase A since exposure to this enzyme for 6 hours failed to liberate any additional amino acids.

(*c*) *Nagarse Digestion of the Octapeptide.*—Nagarse hydrolyzed the octapeptide into 2 tetrapeptides. These were isolated from paper after electrophoresis at *p*H 1.9. The composition of peptide I was Met, Val, Ala, Thr, and that of peptide II was Gly, SCM-cysteine, Ileu, Arg. Edman degradation of peptide II yielded the following results:

1st cycle: PTH glycine; residue: Arg 1, SCM-cysteine 1, Ileu 1, *Gly 0.3.*

2nd cycle: PTH-isoleucine; residue: Arg 1, SCM-cysteine 0.9, Gly 0.3, *Ileu 0.3.*

Hence the sequence of the octapeptide is Met-Val-Ala-Thr-Gly-Ileu-SCM-cysteine-Arg.

DISCUSSION

Thiol groups have long been known to be essential for the catalytic activity of horse liver-alcohol dehydrogenase (Boyer, 1959). We have reported recently that iodoacetate completely abolishes the activity of this enzyme through the preferential and selective carboxymethylation of two of its 28 −SH groups. Since DPN(H) completely prevents both this chemical modification and its enzymatic consequences, these −SH groups must be situated at the active centers (Li and Vallee, 1963).

As a guide to the isolation of the peptide containing the "active" cysteinyl residues, [^{14}C]iodoacetate was reacted with the enzyme until activity was completely abolished; both active-center cysteines were thereby converted to [^{14}C]carboxymethylcysteine. While both trypsin and succinyltrypsin hydrolyzed the modified enzyme readily, succinyltrypsin was employed to achieve sustained proteolytic activity during the long periods of incubation required. Digestion with chymotrypsin, however, was incomplete even when the modified enzyme was denatured with sodium dodecylsulfate.

Virtually all of the ^{14}C activity was localized in one single peptide of the 36 to 38 which were separated by electrophoresis and chromatography of the tryptic digest (Fig. 1). While the enzyme contains a total of 80 lysyl and arginyl bonds[2] susceptible to cleavage

[2] T. K. Li and B. L. Vallee, unpublished results.

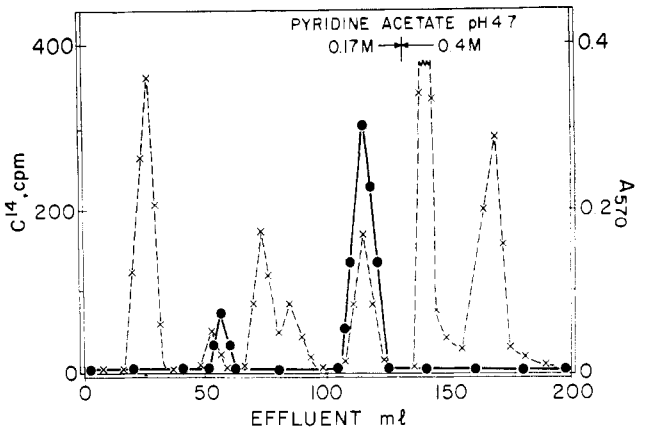

FIG. 3.—Purification of [^{14}C]carboxymethylcysteinyl peptide by chromatography on Dowex 50-X2 (column, 0.9 × 30 cm). The [^{14}C]peptide fractions eluted from Sephadex between 76 and 82 ml were redissolved in 1 ml of 0.17 M pyridine acetate, *p*H 4.7, and applied to the column. Elution was initiated with 0.17 M pyridine acetate, *p*H 4.7, and the buffer was changed to 0.4 M pyridine acetate, *p*H 4.6 after 130 ml. Two-ml fractions were collected at a flow rate of 20 ml/hr. ^{14}C activity (●) and ninhydrin color at 570 mμ (×) were measured as described under Methods. All of the ^{14}C activity, as well as ninhydrin-positive material was eluted in 200 ml.

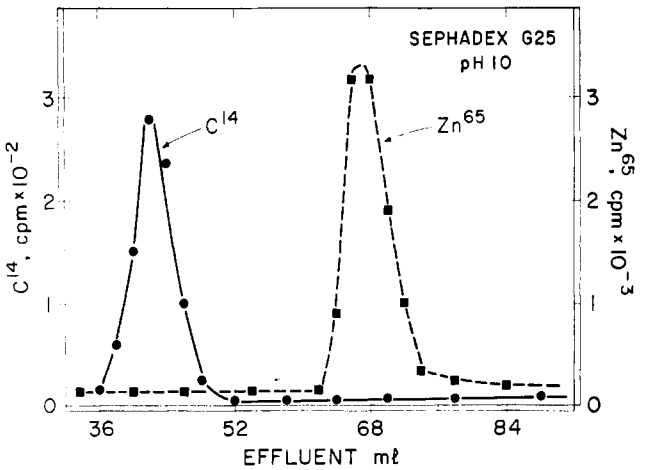

FIG. 4.—Gel filtration of tryptic digests of [^{14}C]carboxymethyl- and ^{65}Zn-labeled liver-alcohol dehydrogenase. The digests dissolved in 2 ml of water were applied to Sephadex G-25 (column, 0.9 × 100 cm) and eluted with water adjusted to *p*H 10 with ammonia. Two-ml fractions were collected at a flow rate of 30 ml/hr. ^{14}C activity (●) was measured as described under Methods and ^{65}Zn activity (■) was measured in a well-type scintillation counter (Coleman and Vallee, 1962).

by trypsin, the number of tryptic peptides found approximates only half this number. This suggests that the enzyme consists of two amino acid chains which are closely similar or identical in amino acid sequence.

When paper electrophoresis and chromatography were employed to isolate the labeled peptide, decomposition of [^{14}C]carboxymethylcysteine occurred resulting in decreased yield. The sensitivity of thioether bonds to air oxidation under acidic conditions has been noted (Crestfield *et al.*, 1963; Moore and Stein, 1963). The anomalous peaks which replaced that of carboxymethylcysteine in the analytical amino acid chromatogram probably represent oxidation products of carboxymethylcysteine.

To obviate these difficulties, gel filtration and column rather than paper chromatography were employed to isolate the peptide. Thiodiglycol[3] was added to the equilibrating and eluting buffers since this agent protects the thioether bond of carboxymethylcysteine against oxidation. Thorough evacuation of the hydrolysis tubes further prevented the decomposition of carboxymethylcysteine during acid hydrolysis. In this manner a single [^{14}C]carboxymethylcysteinyl peptide was isolated. Since two cysteinyl residues per mole of protein were modified, it would appear that they are parts of an identical sequence in the two chains of the enzyme.

The sequence of the cysteinyl octapeptide obtained from the active center of this enzyme differs distinctly from that of other "active-center peptides" isolated thus far. Aside from cysteine the sequence does not contain amino acid residues presently thought to be particularly characteristic participants in catalysis, substrate binding, or both. While cysteinyl residues are known to be essential, their precise role in the catalytic mechanism of this and many other enzymes is still uncertain.

DPN^+ and DPNH both protect the cysteinyl residues against modification by iodoacetate; but the inactive carboxymethylated enzyme still retains the capacity to bind DPNH (Li and Vallee, 1963). Hence, the thiol groups of these particular cysteinyl peptides do not appear to be indispensable for coenzyme binding. The protection of the thiol groups by DPN^+ and DPNH must therefore be attributed to steric factors generated by their proximity to the coenzyme-binding sites of the enzyme.

Thiol groups have also been implicated as the ligand sites for the catalytically essential zinc atoms of this enzyme (Druyan and Vallee, 1962), and it might be inferred that the cysteinyl residue of this peptide is one of the donors of the zinc-ligand site. However, tryptic digestion of [(LADH)$^{65}Zn_2$][4] and separation of the resultant peptides by passage of the digest over Sephadex G-25 under conditions identical to those employed for the [^{14}C]carboxymethylcysteinyl enzyme demonstrates that ^{65}Zn is firmly bound to a peptide; but this material emerges as a single peak which is distinctly separate from the [^{14}C]carboxymethylcysteinyl-containing fraction (Fig. 4). Hence, the ^{65}Zn and ^{14}C labels do not occur in the same peptide and the carboxymethylcysteinyl residues are not responsible for zinc binding. It cannot be stated as yet, however, whether in the primary structure of the enzyme these peptides are contiguous or separated by other amino acid sequences.

Both the isolation and the sequence of this zinc peptide are of great interest since the metal represents its "intrinsic" label, and the peptide would not be readily identifiable on any other basis. Delineation of the position of this peptide in the primary sequence of the enzyme, relative to that of the carboxymethylcysteinyl peptide, is now in progress and would seem to be an indispensable step in the elucidation of the active center of horse liver-alcohol dehydrogenase, the ultimate objective of these studies.

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[3] We are indebted to Drs. T. Y. Liu and W. H. Stein for the suggestion to employ thiodiglycol in this manner.

[4] The designation [(LADH)$^{65}Zn_2$] represents ^{65}Zn-labeled liver-alcohol dehydrogenase in which the two functional zinc atoms at the active center have been replaced with ^{65}Zn by equilibrium dialysis (Druyan and Vallee, 1962).

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