Triphosphopyridine Nucleotide Specific Isocitrate Dehydrogenase from *Azotobacter vinelandii*. Alkylation of a Specific Methionine Residue and Amino Acid Sequence of the Peptide Containing This Residue†

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ABSTRACT: Triphosphopyridine nucleotide (TPN^+) specific isocitrate dehydrogenase from *Azotobacter vinelandii* was inactivated by iodoacetic acid by the modification of a single methionyl residue. This methionyl residue was specifically labeled by [2-^{14}C]iodoacetic acid. Cyanogen bromide cleavage of the carboxymethyl enzyme yielded two ^{14}C-labeled peptides, CNBr I and CNBr II, containing about 193 and 32 amino acid residues, respectively. These peptides contain an overlapping sequence and result from incomplete cleavage of a methionylglycyl peptide bond. The partial amino acid sequence of CNBr II was determined by automatic Edman degradation. The cysteinyl residues were also carboxymethylated by using [2-^{3}H]iodoacetic acid after the enzyme had been denatured in 8 M urea. None of the cysteinyl residues were found in the CNBr peptides which contained the reactive methionine.

Triphosphopyridine nucleotide (TPN^+) specific isocitrate dehydrogenase from *Azotobacter vinelandii* has been previously isolated in this laboratory and its properties were studied (Chung and Franzen, 1969). The high concentration of this enzyme, which accounts for 1–2% of the total soluble protein of the organism, suggests possible key roles of this enzyme in energy production and nitrogen fixation. In addition, sufficient quantities of the enzyme may be readily obtained to permit chemical studies of its structure and function.

This enzyme contains three cysteine residues, one of which is unusually reactive with sulfhydryl reagents. Iodoacetic acid and 5,5′-dithiobis(2-nitrobenzoic acid) each react with equal molar amounts of enzyme with a concomitant loss of catalytic activity (Chung and Franzen, 1969). The reactive cysteine can react reversibly with a second cysteine to form an enzyme species with an intramolecular disulfide bond and which is devoid of enzymatic activity (Braginski *et al.*, 1970). However, enzyme containing the thiocyano derivative of the reactive cysteine exhibited 30–50% of the catalytic activity of the native enzyme (Chung *et al.*, 1971). This suggests that the reactive cysteine is not essential for catalytic activity but that it is probably near the active site. Recently, pyridoxal phosphate was found to modify specifically two–three amino acid residues in the enzyme; this resulted in a 75% loss of enzymatic activity (unpublished observation). Since the pyridoxal phosphate modified enzyme was less reactive toward sulfhydryl reagents, at least one of the modified amino acid residues appears to be near the reactive cysteine residue.

In the present work isocitrate dehydrogenase was carboxymethylated with [2-^{14}C]iodoacetic acid and the sequence of a CNBr peptide containing the carboxymethyl residue was determined. We now report evidence that iodoacetate carboxymethylates a single methionyl residue in the native enzyme, as has been previously demonstrated with TPN-dependent pig heart isocitrate dehydrogenase (Colman, 1967). In addition, the cysteine residues were differentially labeled by carboxymethylation with [2-^{3}H]iodoacetic acid in urea.

Experimental Procedures

Materials. Isocitrate dehydrogenase was prepared as previously described (Chung *et al.*, 1971). Iodoacetic acid was obtained from Sigma Chemical Co. [2-^{14}C]Iodoacetic acid (32 Ci/mol) and [2-^{3}H]iodoacetic acid (113 Ci/mol) were obtained from Amersham/Searle. Cyanogen bromide was purchased from Eastman Organic Chemicals. Trypsin (type XI, diphenylcarbamyl chloride treated) was purchased from Sigma. Maleic anhydride was obtained from Fisher Chemical Co., and urea (Ultra Pure) was obtained from Schwarz/Mann. Sephadex was obtained from Pharmacia.

Preparation of [^{14}C,^{3}H]Carboxymethyl Isocitrate Dehydrogenase. Carboxymethylation was carried out according to the procedure of Crestfield *et al.* (1963). Isocitrate dehydrogenase, 2.69 μmol (215 mg in 18 ml of 0.05 M potassium phosphate buffer (pH 7.0), was flushed with N_2 for 20 min and the pH was adjusted to 7.55 with 0.5 M K_2HPO_4 using a Radiometer pH-Stat. [2-^{14}C]Iodoacetic acid (100 μCi, 3.12 μmol) was dissolved in 300 μl of 0.05 M potassium phosphate buffer (pH 7.0) and was added to the enzyme solution in two equal amounts, at zero time and at 4 hr. The pH was maintained at 7.55 and carboxymethylation was allowed to proceed with stirring at room temperature in the dark. After 20 hr, 10 mg of unlabeled iodoacetic acid (20× molar excess) was added and the reaction was continued for an additional 18 hr. Under these conditions, more than 99% of the enzymatic activity was lost. The [^{14}C]carboxymethyl enzyme was exhaustively dialyzed in the dark for 24 hr with five changes of 0.05 M potassium phosphate buffer (pH 7.0). The enzyme solution was flushed with N_2 and was made 8 M in urea and 0.2% in EDTA-Na_2. The pH was adjusted to 7.5 with 1 μl/ml of 0.6% aqueous methylamine and finally with 0.5 M K_2HPO_4. [2-^{3}H]-

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Iodoacetic acid (1.25 mCi, 11.1 μmol) in 0.1 ml was added to the enzyme solution and, after 1.5 hr, 18 mg of unlabeled iodoacetic acid was added. After 1 hr the enzyme was exhaustively dialyzed for 85 hr with eight changes of distilled water. The fully carboxymethylated enzyme was lyophilized. The incorporation of radioactivity into the enzyme, which was 11.9 μCi of ^{14}C and 111.4 μCi of ^{3}H, indicated that 1 mol of enzyme had reacted with 0.138 mol of [^{14}C] iodoacetic acid and with 0.367 mol of [^{3}H]iodoacetic acid.

CNBr Cleavage of Carboxymethyl Isocitrate Dehydrogenase. CNBr cleavage was carried out according to Steers *et al.* (1965). Glassware was treated with 5% dimethyldichlorosilane in toluene and then rinsed twice with toluene and with methanol (Horning *et al.*, 1963) to reduce adsorption of CNBr peptides. The carboxymethyl enzyme was dissolved in 10 ml of 70% formic acid to which 228 mg of CNBr (100-fold molar excess with respect to methionine residues) had been added and the solution was flushed with N_2 and stored in the dark at room temperature for 16.5 hr. The solution was evaporated to dryness using a rotary evaporator and the peptides were dissolved in 4 ml of 0.2 N acetic acid. After centrifugation 93% of the ^{14}C label and 88% of the ^{3}H label were recovered in the supernatant solution.

Isolation of CNBr Peptides. CNBr peptides were chromatographed in two portions on a Sephadex G-75 (superfine) column (2.5 × 100 cm), which had been equilibrated with 0.2 N acetic acid. The absorbance at 280 nm was determined with a Zeiss PMQ spectrophotometer. Fractions containing radioactive peptides were pooled and evaporated to dryness with a rotary evaporator. The peptides were then chromatographed on a DEAE-Sephadex A-25 column (0.8 × 21 cm) with a linear gradient consisting of 500 ml each of 0.01 M pyridine–*N*-ethylmorpholine–acetate buffer (pH 8.4) and 0.2 M pyridine–acetate buffer (pH 5.0) (prepared according to Schroeder *et al.*, 1962) at a flow rate of 26 ml/hr maintained by a Harvard "Multi-Speed Transmission" pump. The peptides were finally rechromatographed on Sephadex G-75. CNBr peptides were also isolated by chromatography on SE-Sephadex C-25 according to the procedure of Coffee *et al.* (1971). Small CNBr peptides (less than 30 residues) were also chromatographed on a Beckman PA-35 spherical resin using an amino acid analyzer (Benson *et al.*, 1966).

Maleylation of CNBr I and Isocitrate Dehydrogenase and Tryptic Cleavage at Arginine Residues. Lysyl residues were blocked by maleic anhydride as described by Butler *et al.* (1969). To CNBr I (44 nmol, 1.2 × 10^6 dpm of ^{14}C) in 1 ml of 0.2 N sodium borate (pH 9.0) was added 5.9 mg of maleic anhydride dissolved in 0.1 ml of benzene. The reaction was carried out at 4° for 19 hr. An additional 5.9 mg of maleic anhydride was added and the reaction was allowed to continue for 1 hr. The maleyl-CNBr I was isolated on a Sephadex G-50 column, which was equilibrated with 0.05 N $(NH_4)_2CO_3$. The peptide was evaporated to dryness with a rotary evaporator and was digested with 0.0005% trypsin in 2 ml of 0.025 M Tris buffer (pH 8.0).

[^{14}C,^{3}H]Isocitrate dehydrogenase (800 nmol, 11.2 × 10^6 dpm of ^{14}C) was allowed to react with 108 mg of maleic anhydride for 90 min in 18 ml of 0.05 M sodium borate (pH 9.0). The maleylated protein precipitated and was redissolved in 4 ml of 0.05 M Tris (pH 8.0). The protein was digested by the addition of 64 μl of 1% trypsin in 0.001 N HCl.

Isolation of ^{14}C-Labeled Tryptic Peptides Obtained from CNBr I and CNBr II. For these experiments isocitrate dehydrogenase was carboxymethylated as described above except that only the reactive residue was radiochemically labeled. Enzyme (54 mg) was allowed to react with 50μCi of [2-^{14}C]-iodoacetic acid. The specific radioactivity of the modified enzyme was 4.20 μCi/μmol. CNBr I and CNBr II were purified on SE-Sephadex as described above. The peptides were dissolved in 4 ml of glass distilled water and were maintained at pH 9.0 with 0.1 N NaOH using a Radiometer pH-Stat. A 1% trypsin solution was prepared in 0.001 N HCl and assayed before use (Schwert and Takenaka, 1955). Tryptic digestion was carried out at 30° by the addition of 50 μl of the trypsin solution at zero time and at 4 hr. After 23 hr the reaction was stopped by adjusting the pH to 4.5 with glacial acetic acid (Guidotti *et al.*, 1962). The tryptic peptides were desalted on a Sephadex G-10 column, and were then chromatographed on a Sephadex G-25 column which had been equilibrated with 0.2 N acetic acid. The tryptic peptides were further purified by two-dimensional high voltage electrophoresis and paper chromatography. High voltage electrophoresis was carried out in a Camag apparatus af 3000 V, 25 mA, for 1 hr, using pH 4.4 pyridine–acetate–water (1 : 2 : 250) buffer. Paper chromatography was carried out in the second dimension with isoamyl alcohol–pyridine–water (7 : 7 : 6) (Baglioni, 1961). The chromatograms were thoroughly dried and the ^{14}C-labeled peptides were located by the exposure of Ilford Industrial type G X-ray film. The tryptic peptides isolated from Sephadex G-25 were alternatively purified by chromatography on an 0.8 × 60 cm Dowex 50 column (Schroeder, 1967).

Tryptic Peptides of CNBr II. CNBr II was isolated as described above from a separate preparation of [^{14}C,^{3}H]carboxymethyl isocitrate dehydrogenase (^{14}C, 5.83 μCi/μmol). Purified CNBr II (117 nmol) was dissolved in 1 ml of 0.05 M Tris buffer (pH 8.0) and digested for 16 hr at 30° by the addition of 10 μl of 1% trypsin. Tryptic peptides were chromatographed on a 1.5 × 120 cm Sephadex G-25 (superfine) column, which had been equilibrated in 0.2 N acetic acid. The elution of the peptides was monitored by measuring the absorbance at 230 nm in a Cary Model 16 spectrophotometer.

Sequence Analysis. The partial amino acid sequence of CNBr II was determined by automated Edman degradation in a Beckman Model 890C sequencer using a dimethylbenzylamine peptide program. The phenylthiohydantoins obtained from each cycle were identified as such or as the trimethylsilyl derivatives by gas chromatography (Pisano and Bronzert, 1969). The phenylthiohydantoins were also identified by thin-layer chromatography (Jeppsson and Sjoquist, 1967). Arginine and histidine were detected by spot tests, using phenanthrenequinone (Yamada and Itano, 1966) and Pauly's reagent, respectively. The radioactivity of aliquots of the phenylthiohydantoins was measured with a Packard liquid scintillation counter in order to determine the position of the ^{14}C-labeled residue in the sequence. A sample of CNBr II, 160 nmol, was subjected to 23 cycles of Edman degradation. Another sample, 370 nmol, was pretreated with 4-sulfophenyl isothiocyanate (Pierce Chemical Co.) (Inman *et al.*, 1972) and was subjected to 33 cycles of Edman degradation. This procedure enabled more residues in the sequence to be determined, although the N-terminal residue and lysyl residues could not be directly determined. The combination of the two procedures yielded the maximum of sequence information. Amino acid compositions determined for the tryptic peptides obtained from CNBr II were used to confirm sequence data obtained by automatic sequencing and also to determine the remaining positions in the sequence. The carboxyl-terminal peptide, which included the carboxymethyl residue, was subjected to three cycles of manual Edman degradation according to the procedure of Peterson *et al.* (1972).

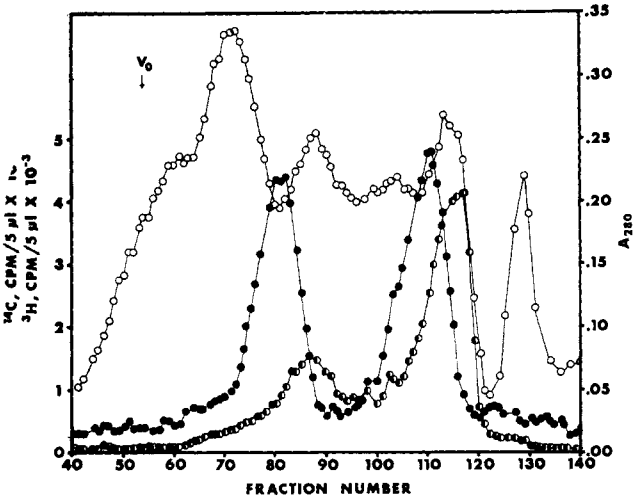

FIGURE 1: Fractionation of CNBr peptides by gel filtration on Sephadex G-75. CNBr-treated [^{14}C, ^{3}H]carboxymethyl isocitrate dehydrogenase (1.35 μmol in 2 ml) was applied to a 2.5 × 100 cm column of Sephadex G-75 (superfine) equilibrated in 0.2 N acetic acid. The flow rate was 8.2 ml/hr and 20-min fractions were collected. V_0 was 147 ml as determined by dextran blue: (●) ^{14}C; (◐) ^{3}H; (○) A_{280}.

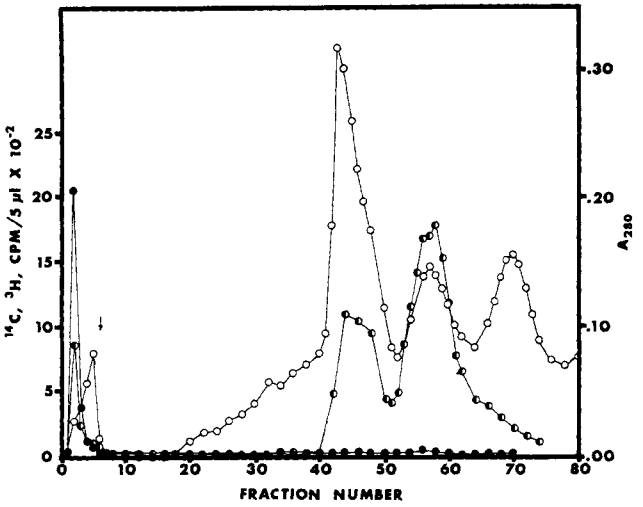

FIGURE 2: DEAE-Sephadex chromatography of CNBr II and CNBr IV. A mixture of CNBr II and CNBr IV, which was obtained by pooling fractions from Sephadex G-75, was applied to a DEAE-Sephadex column. After washing the column for 90 min with starting buffer, the remaining peptides were eluted with a linear gradient (see text). The flow rate was 26 ml/hr and 15-min fractions were collected: (●) ^{14}C; (◐) ^{3}H; (○) A_{280}.

Amino Acid Analysis. Peptide and protein samples were sealed under N_2 in 6 N HCl and were hydrolyzed at 110° for 20 hr, unless otherwise indicated. Hydrolyses were carried out for 72 hr when complete release of valine and isoleucine was desired. Serine and threonine were corrected for losses during hydrolysis; the recovery was 86 and 88%, respectively, for 20-hr hydrolysis. *S*-Carboxymethylcysteine (BDH Chemicals) was subjected to the same conditions of hydrolysis and the recovery was 85%. Constant boiling HCl (108°) was twice distilled. Amino acid analyses were carried out according to the procedure of Spackman *et al.* (1958) using a Beckman amino acid analyzer.

Enzyme Assay. Enzymatic activity was determined as previously described (Chung and Franzen, 1969). Protein concentrations were determined by the absorbance at 280 nm.

Identification of Carboxymethylmethionine. Carboxymethylmethionine was identified by amino acid analysis of hydrolyzed samples. The chromatographic elution patterns of both ninhydrin-positive peaks and of radioactive peaks were compared to those obtained for the authentic derivative. Carboxymethylmethionine was prepared as described by Gundlach *et al.* (1959a). [^{14}C]Carboxymethylmethionine was prepared by the reaction of 25 μmol of L-methionine with 1.8 μmol of [1-^{14}C]iodoacetic acid (13.7 μCi/μmol, New England Nuclear) for 72 hr and then with 54 μmol of unlabeled iodoacetic acid for 27 hr at 40°. The product was heated at 110° for 20 hr under the standard conditions for protein hydrolysis.

Nomenclature. ^{14}C-Labeled CNBr peptides are designated CNBr I and CNBr II by the order of elution from Sephadex G-75. ^{3}H-Labeled CNBr peptides are designated as CNBr III, which elutes after CNBr I, and CNBr IV, which elutes just after CNBr II. Tryptic peptides of CNBr peptides are designated with "T" by the order of elution from Sephadex G-25, *e.g.*, CNBr II-T1, etc. CNBr I and isocitrate dehydrogenase which were treated with maleic anhydride are designated with an "M." Peptides released from M-CNBr I with trypsin are referred to as maleyl peptides (cleavage specifically at arginine) and are designated M-CNBr I-T1, etc. Tryptic peptides obtained from maleyl isocitrate dehydrogenase are referred to as MT-I, etc., in order of elution from Sephadex G-50.

Results

Isolation of CNBr Peptides. Figure 1 shows the elution of the CNBr peptides on Sephadex G-75. Two ^{14}C-labeled CNBr peptides were separated, CNBr I and CNBr II, which eluted at 222 and 304 ml, respectively. A ^{3}H-labeled peak, CNBr III, eluted at 238 ml and was incompletely resolved from CNBr I. CNBr I and CNBr III were resolved on DEAE-Sephadex. A second ^{3}H-labeled peak, CNBr IV, eluted at 318 ml and was incompletely resolved from CNBr II. When fractions containing CNBr II and CNBr IV were pooled and chromatographed on DEAE-Sephadex, CNBr II was not retained and was thus resolved from CNBr IV, which was eluted with a linear gradient (Figure 2). A second ^{3}H peak eluted just before CNBr IV on DEAE-Sephadex. However, this material was not pure, and whether it represented a different form of CNBr IV or another ^{3}H-labeled peptide could not be determined. When fractions which contained these two ^{3}H-labeled peaks were pooled and chromatographed on PA-35 spherical resin, two peaks were also resolved by this column, but amino acid analysis of peak fractions indicated that the two peaks were identical in composition. The yield of CNBr II was 24–32% of the molar quantity of the enzyme used. The yield of CNBr I purified by SE-Sephadex was 37% of the molar quantity of the enzyme used.

Table I shows the amino acid compositions of CNBr I, CNBr II, and CNBr IV, and the tryptic peptides obtained from CNBr II.

The specific radioactivities of CNBr I and CNBr II for ^{14}C were approximately the same as that of the carboxymethyl enzyme. For a preparation of carboxymethyl isocitrate dehydrogenase with a specific radioactivity of 6.37 μCi/μmol, the isolated CNBr II had a specific radioactivity of 5.43 μCi/μmol. CNBr I, which was isolated from a separate preparation of carboxymethyl isocitrate dehydrogenase (10.6 μCi/μmol), had a specific radioactivity of 11.1 μCi/μmol.

TABLE I: Amino Acid Composition of CNBr I, CNBr II, and CNBr IV, and CNBr II Tryptic Peptides.

	Residues/Molecule[a]					
				CNBr II		
Amino Acid	CNBr I	CNBr II	CNBr IV	T1	T2	T3
Lysine	15.6 (16)	4.0 (4)	3.0 (3)	1.9 (2)	0.9 (1)	
Histidine	2.9 (3)	0.9 (1)	1.9 (2)		1.4 (1)	
Arginine	5.1 (5)	1.2 (1)	1.1 (1)			
Carboxymethylmethionine[b]	(1)	(1)				(1)
Carboxymethylcysteine			0.7 (1)			
Aspartic acid	18.5 (19)	3.4 (3)	3.3 (3)	1.8 (2)		
Threonine[c]	8.5 (9)	1.2 (1)	2.0 (2)			1.0 (1)
Serine[c]	13.4 (13)	2.2 (2)			0.6 (1)	
Glutamic acid	40.9 (41)	2.9 (3)	2.4 (2)	2.0 (2)		
Proline	5.3 (5)		1.7 (2)			
Glycine	13.5 (14)	2.0 (2)	2.9 (3)		1.2 (1)	
Alanine	21.0 (21)	5.0 (5)	2.0 (2)	3.0 (3)		1.0 (1)
Valine	10.7 (11)	2.0 (2)	1.6 (2)		2.0 (2)	
Isoleucine	9.8 (10)	1.9 (2)	0.9 (1)	2.0 (2)		
Leucine	14.1 (14)	3.4 (4)	0.9 (1)		3.0 (3)	
Tyrosine	4.0 (4)		1.1 (1)			
Phenylalanine	4.7 (5)	0.8 (1)	0.7 (1)	0.9 (1)		
Homoserine[d]	(2)	(1)	(1)			1.3 (1)
Total[e]	193	33	28	12	9	4

[a] These values are based upon the integral number of alanine residues except for T2, which is based upon leucine. CNBr peptides were hydrolyzed for 20 hr and tryptic peptides were hydrolyzed for 72 hr. [b] Carboxymethylmethionine was identified by its degradation products (see text). [c] The values for threonine and serine were corrected for losses by destruction during hydrolysis, except those obtained for the tryptic peptide samples, which were hydrolyzed for 72 hr. A separate sample of T2 was hydrolyzed for 20 hr and the value obtained for serine was 1.0; however, the values for valine and leucine were 0.7 and 2.0, respectively, indicating incomplete release of these residues. [d] Homoserine was determined as the total of the free amino acid and its lactone. [e] Total figures indicate the sum of the nearest integers determined for each amino acid. Tryptophan was not determined.

[^{14}C]*Tryptic Peptides Obtained from CNBr I and CNBr II.* Since only 1 mol of iodoacetic acid reacts with 1 mol of isocitrate dehydrogenase (Chung and Franzen, 1969), the resulting two ^{14}C-labeled peptides could be explained by either (1) the reaction of iodoacetic acid with either of two separate sites to produce two carboxymethylated species or (2) incomplete cleavage of CNBr of a peptide bond to produce two peptides containing the same carboxymethylated site. In order to test the latter possibility, CNBr I and CNBr II were each subjected to tryptic digestion. The behaviors of the ^{14}C-labeled peptides obtained from CNBr I or CNBr II were similar on Sephadex G-25 chromatography, high-voltage electrophoresis, paper chromatography, and Dowex-50 ion-exchange chromatography.

[^{14}C]*Tryptic Peptide Obtained from CNBr I after Blocking Lysine Reversibly with Maleic Anhydride.* Since CNBr I contains five arginine residues, six tryptic peptides are expected if the peptide is cleaved at each arginine residue. Five peptides were partially reolved on Sephadex G-50, and the three larger peptides were purified by re-chromatography on the same column. Amino acid compositions of these peptides revealed that they contained about 58, 54, an 38 residues, accounting for about 150 of 193 residues of CNBr I. Each peptide had one arginyl residue. All of the ^{14}C label was found in M-CNBr I-T3. The amino acid composition of this peptide was the same as that of CNBr II, except that it contained an additional six residues. This is in agreement with an overlap in the sequences of CNBr I and CNBr II. Furthermore, the single arginyl residue in M-CNBr I-T3 probably results from the noncleavage of an Arg-Lys sequence which was found in CNBr II (see below), and M-CNBr I-T3 would therefore contain no carboxyl-terminal arginyl residue. Therefore, CNBr I probably contains the overlapping sequence of CNBr II at its carboxyl terminus. Thus, the five peptides partially resolved on Sephadex G-50 should account for all of the tryptic peptides obtained from M-CNBr I.

[^{14}C]*Tryptic Peptide Obtained from Isocitrate Dehydrogenase after Blocking Lysine Reversibly with Maleic Anhydride.* The maleyl tryptic peptides obtained from isocitrate dehydrogenase were separated on Sephadex G-50. All the ^{14}C label appeared in a single peak, which eluted at 100 ml. The ^{3}H label eluted in three peaks, containing approximately equal amounts of radioactivity, at 109, 147, and 170 ml. MT-I was purified by chromatography on Sephadex G-75. Amino acid analysis indicated that MT-I contained about 77–81 residues including only one arginine and one methionine residue.

Nature of the Carboxymethyl Residues. The purified [^{14}C]-carboxylmethyl peptides, CNBr I, CNBr II, and MT-I, exhibited no trace of carboxymethylcysteine upon amino acid analysis, whereas all the purified [^{3}H]carboxymethyl peptides exhibited this derivative, in recoveries of 32–78%. This indicated that [2-^{14}C]iodoacetic acid had reacted with an amino acid residue other than cysteine.

A small ninhydrin-positive peak was apparent in the amino acid chromatograms of [^{14}C]carboxymethyl enzyme and the [^{14}C]tryptic peptide obtained from CNBr II. This peak eluted at 78 min, between proline and glycine, the position previously reported for the elution of carboxymethylhomocysteine, a

1 5 10 15
Gly-Lys-Ser-Ala-Leu-Arg-Lys-Phe-Ile-Ala-Ala-Gln-Ile-Glu-Asp-

20 25
Ala-[(Asx)(Lys)(—)]-Gly-Val-Leu-Leu-( )-Val-( )-Leu-Lys-

30
Ala-Thr-CM-Met-Hse

FIGURE 3: Partial amino acid sequence of CNBr II. The sequence was determined with a Beckman Model 890C automatic sequenator. Two samples of peptide were used, with and without pretreatment with 4-sulfophenyl isothiocyanate. Lys-28 and Hse-32 were placed by compositions of tryptic peptides obtained from CNBr II.

decomposition product of carboxymethylmethionine (Gundlach *et al.*, 1959a). This peak was identical in position with that obtained when carboxymethylmethionine was treated with the conditions for hydrolysis and subjected to amino acid analysis. Both methionine and homoserine (and its lactone) were detected in the chromatograms in both hydrolyzed samples of the ^{14}C-labeled peptide and of carboxymethylmethionine. However, homoserine was also present as the carboxyl-terminal amino acid of the CNBr peptides so the amount of homoserine which was contributed by the degradation of carboxymethylmethionine could not be determined.

When fractions from the eluate of the amino acid analyzer column were collected for a sample of the [^{14}C]tryptic peptide, 21% of the recovered radioactivity was found to eluate at the time expected for carboxymethylhomocysteine. Three ninhydrin-negative peaks eluted before aspartic acid, at 14, 20, and 25 min, and accounted for 33, 26, and 16% of the radioactivity, respectively. When [^{14}C]carboxymethylmethionine was exposed to hydrolysis conditions, 46% of the total recovered radioactivity was found in the carboxymethylhomocysteine peak. In addition, radioactivity was found in three ninhydrin-negative peaks, which eluted at times corresponding to those found for the ^{14}C-labeled peptide, and accounted for 16, 29, and 8% of the recovered radioactivity. Two of the peaks presumably represent *S*-methylthioglycolic acid and glycolic acid, which are released concomitantly with homoserine and methionine, respectively, in the degradation of carboxymethylmethionine (Gundlach *et al.*, 1959a). In addition, 4% of the radioactivity of the ^{14}C-labeled peptide hydrolysate was recovered in the position near the methionine peak which probably represents a small amount of ε-carboxymethyllysine arising from limited carboxymethylation of lysyl residues and which is known to elute in this region (Gundlach *et al.*, 1959b).

Sequence of CNBr II. The amino acid sequence of CNBr II determined by automatic Edman degradation is shown in Figure 3. When CNBr II was directly sequenced the amino acid residues 1–11 were determined. After 11 cycles, the yields dropped dramatically, but residues 15 (Glu), 20 (Gly), and 21 (Val) were tentatively identified. Another sample was pretreated with 4-sulfophenyl isothiocyanate in order to reduce losses due to the extractibility of the peptide. All of the residues previously determined were confirmed, except lysine and the amino-terminal glycine, which were allowed to react with 4-sulfophenyl isothiocyanate. In addition, the remaining sequences through residue 30, except 17–19, 24, 26, and 28, were determined. The radioactive derivative was found at residue 31.

The radioactive tryptic peptide from CNBr II was subjected to three cycles of Edman degradation using a manual, subtractive method. The first two cycles were found to be Ala-Thr, and the radioactive residue was found at the third cycle. This sequence overlaps with residues 29–31 of CNBr II

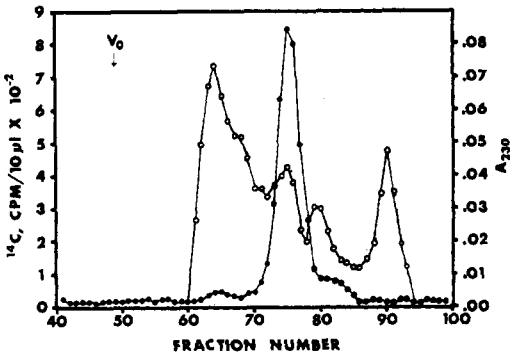

FIGURE 4: Gel filtration chromatography of tryptic peptides obtained from CNBr II. The tryptic digest obtained from 117 nmol of CNBr II was applied in 1 ml to a 1.5 × 120 cm Sephadex G-25 (superfine) column, equilibrated in 0.2 N acetic acid. Chromatography was carried out with a flow rate of 7.9 ml/hr, and 15-min fractions were collected. The absorbancy was determined at 230 nm using a Cary Model 16 spectrophotometer: (●) ^{14}C; (○) A_{230}.

determined by automatic sequence analysis. Therefore, residue 28 must be Lys. The fourth residue was determined by subtraction to be homoserine, indicating that this tryptic peptide is the carboxy-terminal peptide of CNBr II. Furthermore, this places the carboxyl terminus at residue 32, rather than at residue 33, as indicated by the amino acid composition of CNBr II (Table I). The extra residue in the amino acid composition of CNBr II is probably accounted for by the glutamic acid content, which may be falsely elevated by the homoserine content, since homoserine is incompletely resolved from glutamic acid by our amino acid analyzer system. If the glutamic acid content of CNBr II is assumed to be two, the residues thus far identified in the sequence account for all the amino acids in the composition, except His, 2Asx, Lys, and Ser, which must correspond to the five undetermined positions.

Tryptic Peptides of CNBr II. In order to confirm the above sequence data and to determine the positions of the other five residues, tryptic peptides from CNBr II were separated on Sephadex G-25 and amino acid compositions of peak fractions were obtained. Figure 4 shows the chromatographic separation of the expected five tryptic peptides, which are denoted CNBr II-T-1 through CNBr II-T-5 in order of elution. Essentially all of the radioactivity is found in CNBr II-T3. Table I shows the amino acid compositions of CNBr II-T1 through CNBr II-T3, obtained from peak fractions and corrected for overlap of adjacent peptides. CNBr II-T1 contains 12 amino acid residues. Its composition is identical with that for the sequence of residues 8–16, with an additional Asx and 2Lys. CNBr II-T1 was subjected to two cycles of Edman degradation using an automatic sequenator. The amino acid composition of the remaining peptide revealed that the first two residues were Lys and Phe. Therefore, the amino terminus of CNBr II-T1 must be the lysyl residue at position 7 in CNBr II. CNBr II-T2 contains nine amino acid residues and its composition matches the sequence 20–28. One residue each of histidine and of serine are found in this peptide. Therefore, residues 24 and 26 must be histidine and serine, but not necessarily in this order. CNBr II-T3 is the radioactive peptide and is the carboxyl-terminal peptide, as previously determined.

Discussion

In previous studies it was shown that native TPN-specific isocitrate dehydrogenase from *Azotobacter vinelandii* reacted

with iodoacetic acid to produce an inactive enzyme species in which 1 mol of iodoacetic acid was incorporated per mol of enzyme (Chung and Franzen, 1969). Since the enzyme was also inactivated by the reaction of 5,5′-dithiobis(2-nitrobenzoic acid) with a cysteine residue, iodoacetic acid was assumed to inactivate the enzyme by reaction with the same cysteine residue. The results reported here indicate that iodoacetic acid inactivates isocitrate dehydrogenase by reaction with a single methionyl residue. The reactivity of this specific methionyl residue with iodoacetic acid and the resulting loss of catalytic activity suggest that this residue is probably at the active site. However, at present we cannot determine whether this methionyl residue has an essential role in the catalytic mechanism.

We specifically labeled the reactive methionyl residue of the enzyme with [^{14}C]iodoacetic acid, and CNBr cleavage produced two peptides which were then isolated. The following evidence indicates that the two ^{14}C-labeled CNBr peptides contain overlapping sequences with a single ^{14}C-labeled residue. (1) The specific radioactivities of ^{14}C for CNBr I and II are each the same as for the enzyme from which they were isolated. If two or more residues were labeled, the specific radioactivity of peptides containing each labeled residue would be lower than that of the intact enzyme. (2) The ratio of CNBr I to CNBr II is dependent on the conditions for CNBr cleavage. When only a fourfold molar excess of CNBr was used about 80% of the radioactivity was recovered in CNBr I and only 20% in CNBr II. Under the standard conditions of 100-fold molar excess CNBr, about equal amounts of CNBr I and CNBr II were obtained. However, in one experiment in which carboxymethyl enzyme was dialyzed before CNBr cleavage for only 1 day instead of the usual 3 days, ~80% of the radioactivity was recovered in CNBr II. (3) The ^{14}C-labeled tryptic peptides obtained from CNBr I and CNBr II appeared to have similar behavior on gel filtration chromatography, high voltage paper electrophoresis, paper chromatography, and Dowex 50 chromatography. (4) The ^{14}C-labeled tryptic peptide obtained from M-CNBr I had the same composition as CNBr II, with about an additional six amino acid residues. (5) When carboxymethyl isocitrate dehydrogenase is treated with maleic anhydride to block lysyl residues and subsequently digested with trypsin to cleave at arginyl residues, a single ^{14}C-labeled peptide was isolated.

The overlap of CNBr I and CNBr II most probably results from incomplete cleavage of a methionyl peptide bond. Since CNBr II overlaps CNBr I at its carboxyl terminus, the bond resistant to CNBr cleavage must occur between a methionyl residue and the glycyl residue, which is the amino terminus of CNBr II. Many examples of incomplete cleavage of methionyl peptides by CNBr have been reported. Most often the methionine is followed by serine or threonine (Schroeder *et al.*, 1969; DeLange, 1970; Waxdal *et al.*, 1971; Narita and Titani, 1968; Shome *et al.*, 1971; Smith *et al.*, 1970). Incomplete cleavage of other methionyl peptide bonds by CNBr includes the sequences Met-Ile and Met-Glu (Corradin and Harbury, 1970) and Met-Val (Titani *et al.*, 1972). Usually the methionyl residue is converted to homoserine even though cleavage of the peptide bond does not occur. Therefore, absence of methionine in the amino acid composition in a CNBr peptide does not necessarily indicate that it has no intact methionyl peptide bonds.

Amino acid analyses of the ^{14}C-labeled peptides were consistent with ^{14}C carboxymethylation of a methionyl residue. A small ninhydrin-positive peak eluted in the position of carboxymethylhomocysteine, a degradation product of carboxymethylmethionine (Gundlach *et al.*, 1959a). This peak was specifically ^{14}C labeled. In addition, three ninhydrin-negative radioactive peaks eluted before aspartic acid, which are degradation products also characteristic of carboxymethylmethionine.

The finding of a single methionyl residue in the TPN$^+$-dependent isocitrate dehydrogenase which is reactive toward iodoacetic acid suggests a similarity with the TPN$^+$-dependent isocitrate dehydrogenase from pig heart, which also has been found to have a single reactive methionyl residue (Colman, 1967). However, several differences must be noted between our procedures and those employed by Colman in carboxymethylation of the enzyme. In our experiments only a slight excess of [2-^{14}C]iodoacetic acid was used in the initial reaction, to which unlabeled iodoacetic acid was later added to produce a 20-fold molar excess. However, Colman used a 280-fold molar excess of iodoacetic acid. We carried out carboxymethylation at pH 7.55, whereas Colman used pH 5.58. Colman (1968) found that the rate of inactivation by iodoacetic acid decreased markedly above pH 6.0 and showed a slight increase from pH 7.2 to 7.7. However, it was not demonstrated whether inactivation at the slightly alkaline pH was due to alkylation of the methionyl residue or of another residue. We have shown that even at pH 7.55 and with a 20-fold excess of iodoacetic acid, a single methionyl residue was carboxymethylated.

Furthermore, the carboxymethylmethionine derivative was stable to all conditions used for CNBr cleavage (70% formic acid) and peptide purification (0.2 N acetic acid), since no loss in the specific radioactivity was observed between formation of the carboxymethyl enzyme to the isolation of radioactive CNBr peptides and tryptic peptides. Desulfurization to form homoserine, such as Gundlach *et al.* (1959a) found when carboxymethylmethionine was heated at 100°, would lead to a loss of radioactivity. Thus, carboxymethylmethionine in isocitrate dehydrogenase appears to be more stable than the free amino acid. Gundlach *et al.* (1959a) found a similar increased stability of the methionine derivative in ribonuclease. The carboxymethylmethionine appears to decompose only in 6 N HCl under the conditions used for protein hydrolysis.

CNBr cleavage proved to be a useful technique for isolating a peptide which contained the carboxymethylmethionine, since only a few peptides would be produced. Since only gel filtration and DEAE-Sephadex chromatography were required for the isolation of CNBr II, good yields were obtained, even though incomplete cleavage resulted in an almost equal yield of CNBr I. In order to determine the amino acid sequence around the reactive methionine, we wented to obtain a tryptic peptide which could be sequenced by manual Edman degradation. This task was greatly simplified by isolating the tryptic peptide from CNBr II rather than directly from the enzyme in which case many more tryptic peptides would be produced. However, the carboxymethylmethionine was found in the tryptic peptide at the carboxyl terminus of CNBr II. The yield of this peptide greatly diminished after each cycle of Edman degradation but the sequence of this tetrapeptide could be determined by the subtractive method.

We were able to determine nearly the entire sequence of CNBr II using an automatic sequenator. Pretreatment of the peptide with 4-sulfophenyl isothiocyanate (Inman *et al.*, 1972) enabled us to obtain a partial sequence through residue 31, the carboxymethylmethionine, and thus overlapping with the sequence of the tryptic peptide. CNBr II contains a basic Arg-Lys sequence at residues 6–7 and an acidic Glu-Asp sequence at residues 14–15. Residues 31–32 indicate a Met-

Met sequence in the enzyme. Chemical modification of specific residues and the identification of these residues in the amino acid sequence will elucidate some of the structure–function relationships of isocitrate dehydrogenase and will aid in understanding the mechanism of catalysis. Previous studies have indicated that a reactive cysteine may be near the active site. However, we did not find any of the ^{3}H-labeled cysteinyl residues in CNBr I or CNBr II.

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