Nicotinamide Adenine Dinucleotide-specific Glutamate Dehydrogenase of Neurospora

VII. ISOLATION AND SEQUENCES OF THREE LARGE CYANOGEN BROMIDE PEPTIDES*

BRIAN M. AUSTEN and EMIL I. SMITH

From the Department of Biological Chemistry, UCLA School of Medicine, and the Molecular Biology Institute, University of California, Los Angeles, California 90024

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The isolation and sequences of three peptides of large size from a cyanogen bromide digest of the NAD-specific glutamate dehydrogenase of Neurospora crassa are reported. These three peptides comprise 86, 117, and 194 residues, respectively, and represent approximately 30% of the estimated 1030 residues in the peptide chain.

In the preceding papers we have reported the isolation and sequence of tryptic peptides (1, 2) and some cyanogen bromide peptides up to 51 residues in length (3) from the NAD-specific glutamate dehydrogenase of Neurospora crassa. It was apparent from the preliminary fractionation of the cyanogen bromide digest (3) that many large peptides well over 50 residues in length were present. In the investigation of cyanogen bromide digests of other glutamate dehydrogenases (4, 5), it had been difficult to separate such large peptides and it was necessary to extend the use of mixtures of two large peptides with enzymes of different specificity in order to obtain their sequences. It was hoped to develop conditions suitable for chromatography in order to purify such large peptides to homogeneity. In this paper, we report the isolation and sequences of three large cyanogen bromide peptides.

MATERIALS AND METHODS

PTH-derivatives* were identified as described previously (3). PTH-carboxymethylcysteine (PTH-Cys(Cm)) was identified on the thin layer chromatographic system described by Inagami and Murakami (6) with Modified E as solvent. With this solvent, PTH-Cys(Cm) moved away from the origin whereas PTH-Cys(Cm) stayed close to it. Both derivatives gave a similar color with ninhydrin, but only PTH-Cys(Cm) gave an orange fluorescence when the ninhydrinstained spot was viewed under ultraviolet light (>225 nm). Hydrolysis of PTH-Cys(Cm) with 6 M HCl and 2% mercaptoacetic acid yielded alanine and Cys(Cm) in low yield.

Staphylococcal protease hydrolysis of Peptide CN21 was performed with 5% by weight of protease in 0.1 M ammonium bicarbonate at pH 8.1. Since there was a considerable amount of insoluble material that remained after 16 h of hydrolysis at 40°, the solvent was removed, and the residue was dissolved in 1 ml of 98 to 100% formic acid, and then diluted to 40 ml. Ammonium hydroxide was then added to pH 8.1, a further 5% by weight of protease was added, and hydrolysis was continued for an additional 5 h. Some peptides isolated from this digest were obtained in two forms. One type, with slower mobility on electrophoresis at pH 1.9 than its counterpart, was blocked to Edman degradation but could be converted to the other form by incubation with methanolic HCl (7). Ninhydrin-negative Peptide CN21-Sp-TI, Asn-Ala-Arg, obtained by tryptic digestion of one of the staphylococcal protease peptides, yielded equal quantities only of arginine and alanine after hydrolysis with carboxypeptidase A + B, showing that the α-NH₂ group was chemically modified. It was concluded that formylation of the NH₂ group had occurred, probably in anhydrous formic acid. This finding revealed serious drawback to the use of 98 to 100% formic acid as a solvent for peptides.

All other methods and materials have been described in the preceding paper (3). Integral values given for amino acid compositions in all tables are based on the sequences.

RESULTS

The largest peptides obtained from initial fractionation of the cyanogen bromide digest (Fraction 1 of Fig. 2) (3) were pooled. Since it was evident that a large column (2.8 × 150 cm) of Sephacry G-50 in 30% acetic acid was capable of resolving only a limited amount of the total material (3) a different solvent was used.

The elution profile of the large peptides separated on a column of Sephadex G-100 in 6 M guanidine hydrochloride at 40° is shown in Fig. 1. Satisfactory resolution and flow rate were not obtained at lower temperatures. Peaks 11 and 12 were not investigated further since the compositions indicated mixtures of incompletely fragmented very large peptides.

Some of the data are presented as a miniprint supplement immediately following this paper. (Figs. 1 to 4, 6 to 10, 12 and 13 and Tables I through X are found on pp. 8170-8181.) Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Md. 20014. Request Document No. 77M-420, cite author(s), and include a check or money order for $3.75 per set of photocopies.

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Peak 13 material was desalted by dialysis against 20% acetic acid with Spectro-3 membrane, and then chromatographed on CM-cellulose in 8 M urea (Fig. 2). This procedure separated Peptide CN21 (Peaks A and B) from Peptide CN20 (Peak D). Peak C contained a mixture of Peptides CN20 and CN21 as shown by bands obtained by electrophoresis on polyacrylamide in 6.25 M urea at pH 2.7.

Peak 14 material was also desalted and chromatographed on CM-cellulose at pH 3.6 (Fig. 3). Peptide CN19 was obtained from Peak A. The resolution of peptides from Peaks B, C, and D (Fig. 3) and from Peak 15 (Fig. 1) will be discussed later (8). Peak E material (Fig. 3) possessed the same NH₂-terminal sequence as Peptide CN20 on automatic Edman degradation and was not investigated further.

Peptide CN21 - This peptide (Table I), obtained in a yield of 6.2 µmol, showed one predominant band under electrophoresis on polyacrylamide in 6.25 M urea at pH 2.7 (Fig. 4).

Poor yields were obtained upon automated Edman degradation with Program No. 030176 and 0.1 M Quadrol in the coupling step, making unambiguous identification of the PTH derivatives beyond Step 3 impossible. Step 1: PTH-Cys(Cm); Step 2: PTH-His (in the 1 M HCl layer); Step 3: PTH-Asn (T).

Carboxypeptidase A (40°) released equal quantities of homoserine, alanine, aspartic acid, glutamine, and tyrosine, and twice as much phenylalanine, both after 1 h and 16 h. The yield was 20% after 1 h and 45% after 16 h. Since glutamine and phenylalanine residue were later shown to be on the NH₂-terminal side of these 7 residues, the COOH-terminal sequence is (Gln, Phe) (Phe, Tyr) (Ala, Asp, Hse), because aspartic acid is released less readily than alanine, and alanine less readily than tyrosine or phenylalanine. Any other sequence would have yielded unequal quantities of the released amino acids.

The sequence was solved by performing two digests on Peptide CN21, one with trypsin on the maleylated peptide (2.4 µmol) and the other with staphylococcal proteinase (2.7 µmol).

Maleylated tryptic peptides were initially fractionated on Sephadex G-50 (Fig. 6). Fraction 1 (Fig. 6) was demaleylated, then chromatographed on CM-cellulose in 6 M urea at pH 4.9 (Fig. 7). Only Peptide TM2 was retarded by the resin (Fraction D; Fig. 7). Fraction A was rechromatographed on DEAE-cellulose at pH 8 in 6 M urea to yield Peptides TM4 and TM7 in Peaks A and B, respectively (Fig. 8).

Fractions 2 and 3 (Fig. 6) were resolved by preparative electrophoresis at pH 1.9 and chromatography on BPAW. The compositions of the TM peptides are given in Table II, and their locations in the sequence of Peptide CN21 are shown in Fig. 5.

Only Peptide TM1 contained Cys(Cm). The composition indicated that it was derived from the NH₂ terminus of Peptide CN21; this was confirmed by studies on Peptide Sp1. Carboxypeptidases A + B (3 h; 40°) released only arginine from Peptide TM1.

Peptide TM2 was identical with Peptide TM4AB (9), comprising Peptides TIV-J7, TIV-L1, and TIV-L2 (2) of known sequence.

Peptides TM3 and TM5 had been isolated and partially sequenced previously (2) as Peptides TIV-04 and TIV-P4, respectively. Edman degradation of Peptide TM3 yielded: Step 1: PTH-Glu (G, T, R); Step 2: PTH-Glu (G, T, R); Step 3: PTH-Glu + PTH-Gln (T, R); Step 4: residue Glu, 0.9 (0); Thr, 0.9 (1); Gly, 1.0 (1); Leu, 1.0 (1); Pro, 1.0 (1); Arg, 1.0 (1); Step 5: Glu, 0.5 (0); Thr, 0.5 (0); Gly, 1.0 (1); Leu, 1.0 (1); Pro, 1.0 (1); Arg, 1.0 (1). The sequence of Peptide TM3 was completed on Peptides TM3-Sp1 and TM3-Sp2 (Table III). Peptide Sp1, Glu-His-Glu, stained blue with ninhydrin, and was anionic upon electrophoresis at pH 6.5. Thus glutamic acid could be placed at Step 3 of Peptide TM3, and the appearance of PTH-Gln from Edman degradation at Step 3 was undoubtedly due to cleavage of PTH-His at Step 2 during the coupling step. Peptide TM3-Sp2, Glu-Thr-Glu-Leu-Pro-Arg, was neutral at pH 6.5, and did not stain with ninhydrin, thus confirming glutamine at Step 4 of Peptide TM3. The sequence Leu-Pro-Arg at the COOH terminus had been determined previously (2) for Peptide TIV-04; therefore glycine was placed at residue 6 of Peptide TM3, by difference.

Peptide TM4 was identical with Peptide TIII-E (2). Only the position of a threonine residue and an amide residue remained to be determined; the sequence was completed by studies on Peptide Sp10Cl and Sp9 (see below).

DNS-Ser was obtained from Peptide TM6. Aminopeptidase M liberated 1 residue each of tryptophan, aspartic acid, asparagine, and two glutamic acid, in addition to the other residues. The sequences were determined by automatic sequencing (Table IV). Peptide TM6 contained the same residues as Peptide TM5 plus an additional arginine residue. The additional residue was COOH-terminal in Peptide TM6 was shown by hydrolysis with carboxypeptidase A + B (4 h; 40°); Arg, 1.9; Ile, 1.0; Lys, 0.7.

Edman degradation of Peptide TM7 yielded: Step 1 (not identified); Step 2: PTH-Val (G, R); Step 3: PTH-Leu (G, R); Step 4: PTH-Ala (G, R), which showed that Peptide TIV-C (2) represented the NH₂-terminal sequence of Peptide TM7.

Peptide TM8 yielded Arg, 0.9; Tyr, 0.9; and Leu, 0.9, after hydrolysis with carboxypeptidases A + B (3 h; 40°). Edman degradation gave: Step 1: PTH-Val (G, R); Step 2: PTH-Pro (G, R); Step 3: PTH-Asp (G, R). No residue was identified at Step 4, but aminotripeptidase released tyrosine from the residual peptide, placing serine at the fourth step.

Peptide TM9 was previously isolated and sequenced as Peptide TIV-L7 (2).

Peptide TM10, containing homoserine, was extremely hydrophobic; only 2 residues could be identified by Edman degradation before the peptide had extracted into the organic phase. Step 1: PTH-Phe (G, T, R); Step 2: PTH-Val (G, R).

Staphylococcal protease peptides from Peptide CN21 were initially fractionated on a column of Sephadex G-50 in 30% acetic acid (Fig. 9). Peptides in Fractions 4 and 5 were purified by paper electrophoresis and chromatography. Peptide Sp8 was isolated from Fraction 3 by chromatography on a column (1 x 50 ml) of Sulfopropyl (SP)-Sephadex in 30% acetic acid by elution with a linear gradient of 0.01 M sodium acetate (200 ml) and 0.4 M NaCl (200 ml). Compositions of Sp peptides is given in Table V.

The composition of Fraction 1 (Fig. 9) suggested that it contained a large segment of the undigested COOH terminus of Peptide CN21 (Peptide Sp10). Edman degradation released residues from Peptide Sp10 only after incubation with pyrrolidone carboxylic acid peptidase. The first step after this treatment yielded some a-aminobutyric acid after regeneration of the PTH-derivative. This suggested that Peptide Sp10 had been formed by cleavage at the -Glu-Gln- sequence of Peptide TM3.

Fraction 2 (Fig. 9) also appeared to contain a smaller segment of the undigested COOH-terminal region of Peptide CN21. Since Edman degradation gave 2 residues at several steps, it was apparent that at least two peptides in Fraction 2...
FIG. 5. The sequence of Peptide CN21 showing the sequences of previously isolated tryptic peptides above the sequence, designated T, and below the sequence, the peptides isolated from Peptide CN21 after hydrolysis with trypsin after maleylation (TM), with staphylococcal proteinase (Sp) or chymotrypsin (C). Arrows indicate assignments from the NH₂-terminal end (↓) by pyroglutamyl carboxypeptidase (PCP), by Edman degradation, or aminotripeptidase (AT), and from the COOH-terminal end (↑) by carboxypeptidases A, B, or Y.

Fractions 1 and 2 (Fig. 9) were pooled, hydrolyzed with chymotrypsin, and the mixture fractionated on Sephadex G-25 (Fig. 10). Fractions 2 and 3 from this column (Fig. 10) were purified on BPAW to yield Peptides Sp2-C4 and Sp10-C2, respectively. Fractions 4 to 7 were each resolved by electrophoresis on pH 1.9. The compositions of the isolated chymotryptic peptides and the fractions from which they were derived are given in Table VI.

The composition of Peptide Sp1 (Table V) showed that it contained the residues of Peptide TM1, plus the 5 NH₂-terminal residues of Peptide TIV-J7 (2). Thus, Peptide TM1 originated from the NH₂ terminus of Peptide CN21, and its sequence, Cys-His-Asn-Ser-Arg, could be deduced, since the NH₂-terminal 3 residues of Peptide CN21 were known. Poor yields during Edman degradation of Peptide CN21 could have been due to cyclization of the NH₂-terminal S-carboxymethylcysteine residue (10), or to an α-β shift at the Asn—Ser bond. Peptide Sp2 contained the residues of the COOH terminus of Peptide TIV-J7, plus an additional glutamic acid residue at its COOH terminus. Peptide Sp3 was placed at the COOH terminus of Sp2 on the basis of the overlapping Peptide TIV-L1 (2), Glu-Val-Gln-Asn-Lys. Peptide Sp3 contained isoleucine, Glx, and Asx, plus the 4 COOH-terminal residues of Peptide TIV-L1. Edman degradation of Peptide Sp3 yielded: Step 1: PTH-Val (G,R); Step 2: PTH-Gln (T,R). Hydrolysis of Peptide Sp3 with a mixture of carboxypeptidases A and Y (25; 16 h) yielded 1 residue each of aspartic acid, glutamine, isoleucine, lysine, and asparagine. Thus, Peptide Sp3 was formed by staphylococcal protease cleavage at an aspartyl bond. Since the residues of Peptide TIV-L2 (2), Ile-Gln-Asp-Asn-Ala-Arg, overlapped the 3 COOH-terminal residues of Peptides Sp3, Ile-Gln-Asp, Peptide Sp4 overlapped TIV-L2.

Tryptic hydrolysis of Peptide Sp4 yielded Sp4-T1 (Asn-Ala-Arg) and Sp4-T2 (Leu-Glu) (Table IV). Peptide TM69-K1 (9),
Leu-Glu-Phe-Glu-Ala-Ile-Trp-Arg, provided overlaps for Peptides Sp4, Sp5 (Phe-Glu), and Sp6 (Ala-Ile-Trp-Arg-Glu). Edman degradation of Peptide Sp6 released PTH-Ala at Step 1. The tryptophan-containing octapeptide was not isolated from the trypsin digest of the maleylated Peptide CN21, presumably because of its insolubility. Peptide TM3 (Glu-His-Glu-Gln-Thr-Gly-Leu-Pro-Arg), the only TM peptide from Peptide CN21 with NH2-terminal glutamic acid, must follow the octapeptide. The dipeptide His-Glu (Sp7) was also isolated.

The composition of Peptide Sp8 showed that it overlapped the COOH-terminal 6 residues of Peptide TM3 and the NH2-terminal 16 residues of Peptide TM4. Although Peptide TM4 was not obtained in completely pure form from Peptide CN21, it had been previously isolated pure in good yield as Peptide TM4 AAC (8) and a Peptide TIH E (2). Evidence was obtained earlier (2) for identification of all residues from 1 to 18, except for residues 12 and 13. Hydrolysis of Peptide Sp8 with carboxypeptidase A at pH 5.5 (4 h; 40°C) released equal quantities of glutamic acid, aspartic acid, and leucine, confirming the residues identified by automated Edman degradation of Peptide TM4 at Steps 14, 15, and 16.

Peptide Sp9, Asp-Leu-Gln-Arg-Ser-Glu, overlapped Peptides TM4 and 5. Edman degradation yielded: Step 1: PTH-Asp (G,T,R); Step 2: PTH-Leu (G,R); Step 3: PTH-Gln (T,R). Aminotripeptidase hydrolysis of the residue yielded arginine, confirming the results of hydrolysis by carboxypeptidases A + R of Peptide TM4, which gave equal amounts of arginine, glutamine, and leucine. Residues 12 and 13 of Peptide TM4 were placed by automated Edman degradation of Sp10-C1. The major residues identified were: Step 1: PTH-Ala (G,T,R); Step 2: PTH-Ile (G,R); Step 3: PTH-Thr (R). Thus, threonine was located at Step 12 of Peptide TM4, and serine at Step 13, chymotryptic cleavage having occurred principally at leucine-9 of Peptide TM4 during cleavage of the analogous residue in overlapping Peptide Sp10.

The overlap between Peptides TM6 and TM7 was obtained from Peptide Sp10-C9 (Table VI). Edman degradation yielded PTH-Asp (G,T,R) at Step 1, showing that chymotryptic digestion had occurred after tryptophan. Although Peptide TM7 was not isolated in pure form, it was isolated joined to Peptide TM8 as Peptide TM5-B (9). In addition, the two component peptides had also been isolated from the tryptic digest as Peptides TIV-C and TIV-H4 (Fig. 5) (2). Peptide TIV-H4 had been sequenced previously, whereas Peptide TIV-C had been sequenced except for 3 residues at the COOH-terminal end: -(Leu-(Leu,Ile,Asn)-Lys). The compositions of Sp10-C3 and Sp10-C4 confirmed the occurrence of the residues of Peptide TIV-C within Peptide CN21. Peptides C3 and C4, contained the same residues, except for an additional leucine residue placed at the COOH terminus of C3, since Edman degradation of both Peptides C3 and C4 yielded PTH-Ala (G,R).

Peptide Sp10-C8 yielded PTH-Ile (G,R) at Step 1, and PTH-Asn (T,R) at Step 2, confirming the overlap of Peptides TIV-C and TIV-H4, and also completing the COOH terminal sequence of Peptide TIV-C as Leu-Leu-Ile-Asn-Lys. The compositions of Peptides Sp10-C5, and Sp10-C6 completed the overlaps of Peptides TM7, TM5, and TM9, whereas those of Peptides Sp10-C8 and Sp10-C9 overlapped onto Peptide TM10.

The COOH-terminal 16 residues of Peptide CN21 had been isolated previously as part of overlapping Peptide TIV-E, but its sequence was unknown (2). Its composition was however, distinctively high in phenylalanine and serine, and it contained a residue of methionine (2). Peptides Sp10-C10 and Sp10-C11 aided completion of the sequence. Aminotripeptidase released valine from Peptide Sp10-C10, and carboxypeptidase A, only tyrosine. Edman degradation of Peptide Sp10-C11 yielded: Step 1: PTH-Glu (T,R); Step 2: PTH-Asp (T,R); Step 3: PTH-Gly (T,R); Step 4: PTH-Ser (G); Step 5: no residue identified; Step 6: PTH-Pro (G,R). Inasmuch as carboxypeptidase A at pH 8 (6 h; 40°C) released equal quantities of only phenylalanine and glutamine, serine residues could be placed at residues 5 and 7 of Peptide Sp10-C11. The 2 residues identified at the NH2 terminus at Peptide TM10 (Phe-Val) were sufficient to order Peptides C8, C9, and C10.

The COOH-terminal 5 residues of Peptide CN21 were not isolated from the chymotryptic digest of Peptide Sp10, but location of phenylalanine and glutamine at the COOH terminus of Peptide Sp10-C11 permitted placing, from the results of carboxypeptidase action on Peptide CN21, all residues except for one tyrosine and one phenylalanine.

Subtraction of residues contained in the COOH-terminal 18 residues of Peptide CN21 from the composition of Peptide TIV-E (2) leaves Ser-Lys. In addition, a related peptide containing an additional arginine residue at the COOH terminus of Peptide TIV-E (2) was isolated from the trypsin digest of the maleylated protein (Peptide TM61A) (9). Thus, these tryptic peptides overlapped Peptides CN21 and CN2 (Ser-Lys-Arg-Met) (11), and placed Peptide CN21 close to the COOH terminus of the protein (11).

Peptide CN20 — Fraction D (Fig. 2) gave one major band, and two minor bands, after electrophoresis on polyacrylamide gel (Fig. 4). The relative intensity of the two minor bands increased after storage of Peptide CN20 (yield, 4.5 μmol) in 30% acetic acid for several months, and later sequence studies showed that breakdown was due to acid-catalyzed hydrolysis of an Asp—Pro bond.

Automated Edman degradation of Peptide CN20 permitted identification of PTH-derivatives up to residue 57 (Table VII). These results permitted placing known tryptic peptides (Fig. 11).

To complete the sequence, a trypsin digest of maleylated Peptide CN20 was prepared. The demaleylated peptides were chromatographed on a column of Sephadex G-50 (Fig. 12). Peptides in Fractions 2 and 3 were purified by electrophoresis at pH 1.9, and those in Fractions 4 and 5 by electrophoresis at pH 1.9, followed by chromatography on BPAW. Fraction 1 contained Peptide TM11. The compositions of the TM peptides are given in Table VIII.

The composition of Peptide TM1 corresponded to the 9 NH2-terminal residues obtained by Edman degradation and completed the sequence of Peptide TII-A1 (2), which established the overlap of Peptides CN13/14 and CN20 (11). The composition of Peptide TM2 placed histidine at residue 19 of Peptide CN20. The compositions of Peptides TM3, 4, 5, 6, and 7 confirmed the results of Edman degradation between residues 15 and 54, which also permitted location of the previously unknown amide residues of Peptide TIV-H2 (2), now obtained as Peptide TM7 from Peptide CN20. The results on residues 15 to 54 also confirmed the sequence determinations previously performed on Peptides TIV-K1, TIV-08, TIV-N9, TIV-L21, and TIV-H2 (2).

Edman degradation of Peptide TIV8 yielded: Step 1: PTH-Lys (R); Step 2: PTH-Ile (T,R); Step 3: PTH-Lys (R); Step 4: PTH-Asp (R). The first 3 residues agreed with results obtained at Steps 55, 56, and 57 of the Edman degradation of Peptide CN20, which showed that Peptide TIV8 followed TM7.

Peptide TIV8 was hydrolyzed with trypsin, and the three resulting peptides were separated by electrophoresis at pH
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Fig. 11. Relationships of tryptic (TM) obtained from maleylated Peptide CN20 and tryptic peptides (T) obtained from the protein.

1.9 (Table VIII). Peptide TM8-T1 had been isolated, Lys(PLP)-Asn-Lys, as Peptide TIV-022 (1). In addition, Asn-Lys, as Peptide TV-H1 (2), was isolated, presumably because of incomplete modification by pyridoxal phosphate, and hydrolysis at the unaltered lysine residue. The overlapping Peptide TIV-01 (Asn-Lys-Asp-Ile-Pro-Glu-Gly-Gly-Ser-Lys) had been sequenced previously (2), and clearly contained the residues of Peptide TM8-T2 plus Asn-Lys at the NH₂-terminus. Peptide TM8-T3 did not contain lysine. Its composition showed that it contained the partially sequenced NH₂-terminal 6 residues of Peptide TIV-F2 (2). Cleavage at an Asp-Pro bond within Peptide TIV-F9 would yield the residues of Peptide TM8-T2, plus Pro-Lys, a dipeptide, previously isolated as Peptide TIV-06 (2). The residues of Peptide TM8-T2 and TM8-T3 had also been isolated in a fluorescent peptide modified by pyridoxal phosphate (TIII-A2: Asp-Ile-Pro-Glu-Gly-Gly-Ser-Lys(PLP)-Gly Val Ile Leu Leu Lys(PLP)-Asp) (3), which had been formed by acid hydrolysis at the Asp-Pro bond. The fragments in this part of the sequence are shown in Fig. 11.

Since Pro-Lys are the NH₂-terminal 2 residues of Peptide CN16 (3), it seemed likely that the COOH-terminal 46 residues of Peptide CN20 were also those of Peptide CN16, which had been isolated by cleavage of an Asp-Pro bond at its NH₂-terminus. This was confirmed by isolation of Peptide TM9, comprising the first 6 residues of Peptide CN16 (Pro-Lys-Gln-Gln-Asp-Arg) (Table VIII), Peptide TM10 (His-Arg), and Peptide TM11, comprising the remaining 38 residues of Peptide CN16. The composition of Peptide TM11 was sufficient to establish its identity, and no further sequence work was required to complete the sequence of Peptide CN20.

Peptide CN19—Peak A (Fig. 3) gave one rather broad band upon electrophoresis on polyacrylamide (Fig. 4). The broad appearance of the band may have been due to changes occurring during prolonged storage in 30% acetic acid.

Automatic sequencing allowed identification of many residues as PTH-derivatives up to Step 57 (Table IX). Phenylalanine was also identified as its dansyl derivative at the NH₂-terminus. Residual peptide appeared to be extracting into the butyl chloride after Step 24, as shown by the amount of amino acids after hydrolysis of the PTH-derivatives. This caused problems with identification of some residues.

To complete the sequence, Peptide CN19 was hydrolyzed with chymotrypsin, and the peptides were fractionated on a column of Sephadex G-25, followed by electrophoresis at pH 1.9. Compositions of peptides are given in Table X, and the relationships of these peptides, and of peptides isolated from the tryptic digests of the unacylated and maleylated proteins are shown in Fig. 14.

The compositions of Peptides C1, C2, and C3 confirmed the results of the Edman degradation on the first residues. The composition of Peptide TIII-B1 (2) agreed with the sum of the first 18 residues of Peptide CN19, plus the COOH-terminal 7 residues of Peptide CN17. Thus, Peptide CN19 follows Peptide CN17 in the protein subunit.

The composition of Peptide C4 allowed a serine residue to be placed at residue 26. The identity of residues around this region (Steps 25 to 29) was confirmed also by the composition of a peptide, Thr-Ser-Ile-Pro-Tyr, isolated from 52-residue Peptide TM1-AD obtained from the maleylated protein (9).

Hydrolysis of Peptide C5 with aminopeptidase M released 1 residue each of glutamic acid and asparagine, as well as other residues, confirming the PTH-Glu identified as residue 32, and PTH-Asn as residue 35.

Edman degradation of Peptide C6 yielded PTH-Ile (G.R). Aminopeptidase M released 1 residue each of Asp, Glu, Thr, Ala, Lys, Leu, and Ile, allowing Steps 38 and 41 to be filled with Ile and Asp, respectively. Peptide C7 was related to known Peptide TV-N7 (2). Residues identified in Peptide CN19 at Steps 44, 47 through 50, 53 and 54 showed that residues 44 to 55 agreed with the sequence of Peptide TV-N7. Moreover, 46-residue Peptide TM1-AC (9) formed by hydrolysis at the arginine residue within Peptide TV-N7 had Leu-Glu-Ala-Gly-Cys at its NH₂-terminal sequence. Inasmuch as Peptide TM1-AC was completely sequenced and included...
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FIG. 14. Relationships of the peptides obtained by chymotryptic (C) hydrolysis of Peptide CN19. Peptides obtained from the protein by trypsin hydrolysis (T), and by trypsin hydrolysis of the maleylated protein (TM) are shown above the sequence.

the COOH-terminal 41 residues of Peptide CN19, plus the NH₂-terminal 5 residues of Peptide CN21, no attempt was made to purify all the chymotryptic peptides from this portion of Peptide CN19. However, in confirmation of the overlap, the compositions of Peptides C8, C9, and C10 fitted the sequence that was derived from studies of Peptide TM1-AC (9).

DISCUSSION

The three large peptides which are described in this paper represent together approximately 30% of the estimated 1030 residues in the peptide chain of the dehydrogenase. It should be noted that the sequences were determined on yields of 3.4 μmol (86 residues, Peptide CN19), 4.5 μmol (117 residues, Peptide CN20), and 6.2 μmol (134 residues, Peptide CN21). The sequence work was greatly aided by the prior study of the smaller tryptic peptides (1, 21, and the concurrent and later study of the tryptic peptides from the maleylated protein (9). Thus the sequenator results on Peptides CN19 and 20 served to place in sequence many smaller peptides whose sequences had already been determined.

Unfortunately, the sequenator did not yield sufficient results on Peptide CN21 to aid in this respect and it was necessary to study two types of proteolytic hydrolysates to obtain the sequence of this peptide of 134 residues. It is important to note that the staphylococcal protease was a valuable tool in obtaining peptides for overlapping the tryptic peptides from maleylated Peptide CN21. The unique specificity of this bacterial enzyme (12-14) in hydrolyzing mainly at glutamyl, and only rarely at aspartyl, gave fewer and more useful fragments than would have been obtained by using other well known proteinases.

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