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Complete Amino Acid Sequence of Azotoflavin, a Flavodoxin from *Azotobacter vinelandii*†

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ABSTRACT: Amino acid sequence studies which have led to the sequence determination of the *Azotobacter vinelandii* flavodoxin (azotoflavin) are presented in this report. The total sequence of the flavoprotein is of interest since it is the first group II flavodoxin whose sequence has been determined completely. The amino acid sequence of azotoflavin, a flavin mononucleotide (FMN) redox protein, is: H_2N-Ala-Lys-Ile-Gly-Leu-Phe-Phe-Gly-Ser-Asn-Thr-Gly-Lys-Thr-Arg-Lys-Val-Ala-Lys-Ser-Ile-Lys-Lys-Arg-Phe-Asp-Asp-Glu-Thr-Met-Ser-Asp-Ala-Leu-Asn-Val-Asn-Arg-Val-Ser-Ala-Glu-Asp-Phe-Ala-Gln-Tyr-Gln-Phe-Leu-Ile-Leu-Gly-Thr-Pro-Thr-Leu-Gly-Glu-Gly-Glu-Leu-Pro-Gly-Leu-Ser-Ser-Asp-Cys-Glu-Asn-Glu-Ser-Trp-Glu-Glu-Phe-Leu-Pro-Lys-Ile-Glu-Gly-Leu-Asp-Phe-Ser-Gly-Lys-Thr-Val-Ala-Leu-Phe-Gly-Leu-Gly-Asp-Gln-Val-Gly-Tyr-Pro-Glu-Asp-Tyr-Leu-Asp-Ala-Leu-Gly-Glu-Leu-Tyr-Ser-Phe-Phe-Lys-Asp-Arg-Gly-Ala-Lys-Ile-Val-Gly-Ser-Trp-Ser-Thr-Asp-Gly-Tyr-Glu-Phe-Glu-Ser-Ser-Glu-Ala-Val-Val-Asp-Gly-Lys-Phe-Val-Gly-Leu-Ala-Leu-Asp-Leu-Asp-Asn-Gln-Ser-Gly-Lys-Thr-Asp-Glu-Arg-Val-Ala-Ala-Trp-Leu-Ala-Gln-Ile-Ala-Pro-Glu-Phe-Gly-Leu-Ser-Leu-COOH. This single polypeptide chain protein consists of 179 amino acids and contains a single cysteine residue at position 69. Dimerization of two azotoflavin molecules by disulfide bond formation results in the inactivation of azotoflavin as an electron carrier. Azotoflavin differs from previously studied flavodoxins in that the two residues flanking the planar FMN ring are a glutamic acid and a tyrosine residue. Sequence and conformational comparisons of the various group I and group II flavodoxins suggest that the major difference between these two types is a lengthening of the COOH-terminal region of the group II flavodoxin, which further suggests that all of the flavodoxins have arisen from the same ancestral precursor.

Azotoflavin is a naturally occurring FMN[1] redox protein which was isolated from the obligate aerobe *Azotobacter vinelandii.* Since this flavoprotein was first reported as an electron carrier in *Azotobacter vinelandii* (Shethna et al., 1965, 1966), it has been known by the names "Shethna flavoprotein" (Edmondson and Tollin, 1971b), "*Azotobacter* free-radical flavoprotein" (Hinkson and Bulen, 1967), and "Azotoflavin" (Benemann et al., 1969). Earlier work by Hinkson and Bulen (1967), Benemann et al. (1969), and also Cusanovich and Edmondson (1971) had shown that the Shethna flavoprotein would not replace ferredoxin in the photosynthetic reduction of $NADP^+$ by spinach chloroplasts or in the phosphoroclastic assay of extracts from *Clostridia.* This flavoprotein could, however, replace ferredoxin in assays with *Azotobacter* nitrogenase (Benemann et al., 1969), and more recent studies by van Lin and Bothe (1972) have demonstrated that at high concentrations and under anaerobic conditions it could also replace ferredoxin in NADP reduction by illuminated spinach chloroplasts or by molecular hydrogen and hydrogenase from *Clostridium pasteurianum.* Thus, the Shethna flavoprotein was classified as a flavodoxin. Azotoflavin (*Azotobacter* flavodoxin) differs from other flavodoxins

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[1] Abbreviations used are: FMN, flavin mononucleotide; NADP, nicotinamide adenine dinucleotide phosphate.

in that it is a constitutive component of the cell (Yoch and Valentine, 1972 and references cited therein), whereas flavodoxins from other organisms are induced by iron deficiency. The primary function of the flavodoxin in *Azotobacter* appears to be its involvement in the transport of electrons to nitrogenase (Benemann et al., 1969, 1971); however, an endogenous enzyme or enzyme system has yet to be demonstrated which will reduce this flavodoxin (see discussion in Yates and Jones, 1974).

The importance of flavoproteins as redox proteins has been well documented. Yet, the only flavoprotein whose complete three-dimensional structure is known is flavodoxin (Watenpaugh et al., 1972, 1973; Andersen et al., 1972; Burnett et al., 1974). Flavodoxins are low-molecular-weight FMN proteins which are found in various bacteria and algae (Fox et al., 1972) and have not yet been found in plants and animals (Mayhew and Ludwig, 1975). Depending on the criteria used, flavodoxins can be classifed in two ways: (1) the "*pasteurianum* type" and the "*rubrum* type", are suggested by differences in the physicochemical properties such as circular dichroism and fluorescence excitation spectra and FMN binding equilibrium constants of the apoflavodoxins (D'Anna and Tollin, 1972), and (2) "group I" and "group II" flavodoxins due to differences in molecular weight (Tanaka et al., 1975). The nonphotosynthetic bacteria types have molecular weights of about 15 000 and are classified as group I, while the photosynthetic bacteria and algae types have molecular weights of about 22 000 and are classified as group II. With a molecular weight of approximately 20 000, azotoflavin is a group II flavodoxin.

In this paper, the complete amino acid sequence of *Azotobacter* flavodoxin (azotoflavin) is presented and the conformational comparisons of the group I and II flavodoxins are described. A preliminary report of the sequence of the protein has been published (Tanaka et al., 1975).

Experimental Section

Materials

Azotoflavin and Its Derivatives. The isolation and purification of azotoflavin from a pure culture of *Azotobacter vinelandii* strain OP (Berkeley) and the procedures for the preparation of the carboxymethyl derivative of azotoflavin and for the preparation of the succinyl derivative of the carboxymethylazotoflavin have already been described (Tanaka et al., 1975).

Enzymes. Trypsin, chymotrypsin, carboxypeptidase A, and carboxypeptidase B were obtained from the Worthington Biochemical Corp. Both trypsin and α-chymotrypsin were three-times crystallized, salt-free preparations. Prior to use, trypsin and chymotrypsin were treated, respectively, with L-1-tosylamido-2-phenylethyl chloromethyl ketone (Wang and Carpenter, 1965) and L-1-tosylamido-2-lysylethyl chloromethyl ketone (Mares-Guia and Shaw, 1963). Carboxypeptidase A (41 units/mg) and carboxypeptidase B (170 units/mg) were diisopropyl phosphofluoridate treated products. Three-times crystallized thermolysin (A grade) was purchased from Calbiochem.

Other Chemicals. All of the Sequencer reagents of sequanal quality were purchased from Pierce Chemical Co. All of the other reagents were reagent grade and of the highest quality commercially available.

Methods

Amino Acid Analyses and Sequence Analyses. Purified samples of the protein and peptides were hydrolyzed in three-times distilled HCl in vacuum-sealed Pyrex glass tubes at 110 °C. The amino acid compositions were determined in a Beckman-Spinco 120C automatic amino acid analyzer as described by Spackman et al. (1958); the instrument was equipped with high-sensitivity cuvettes and a 4-5-mV full-scale range card.

Automatic sequence analyses (Edman and Begg, 1967) in a Beckman-Spinco Model 890 Protein/Peptide Sequencer were carried out to sequence the carboxymethyl-protein (200 nmol) and peptides which contained more than 25 amino acid residues (100–300 nmol). For protein sequencing, the protein double-cleavage program and 1.0 M Quadrol as the coupling buffer were used, while the protein single-cleavage program and 0.2 M Quadrol coupling buffer were used for the peptide sequencing. Sequence analyses of all the other peptides (100–250 nmol) were carried out by manual Edman degradation procedure (Edman, 1956). In some cases, samples containing lysine residues at their carboxyl-terminal ends were reacted with 4-sulfophenyl isothiocyanate (Braunitzer et al., 1970) prior to the sequence analyses. The amino acid phenylthiohydantoins were identified either by gas chromatography in a Beckman GC-45 gas chromatograph as described by Pisano and Bronzert (1969), thin-layer chromatography on silica gel containing 5% $CaSO_4$ and an ultraviolet fluorescent indicator as described by Edman and Begg (1967), or by amino acid analysis of the 6 N HCl hydrolysates (20 h at 150 °C) of the amino acid phenylthiohydantoins as described by Van Orden and Carpenter (1964).

Enzymatic Hydrolyses and Purification of Peptides. Strategy of sequence determinations of *Azotobacter* flavodoxin (azotoflavin) is summarized in Table I, while the detailed procedures used for these enzymatic hydrolyses have been described in a previous report (Tanaka et al., 1975).

Thermolysin hydrolysis of tryptic peptide T10, which was obtained by trypsin digestion of carboxymethylazotoflavin, was carried out as follows. Thermolysin (enzyme to substrate was 1:20, w/w) was added to the tryptic peptide T10 (0.5 μmol) which was dissolved in 0.5 mL of deionized water (adjusted to pH 8 with *N*-ethylmorpholine) and the digestion reaction was carried out for 18 h at 40 °C.

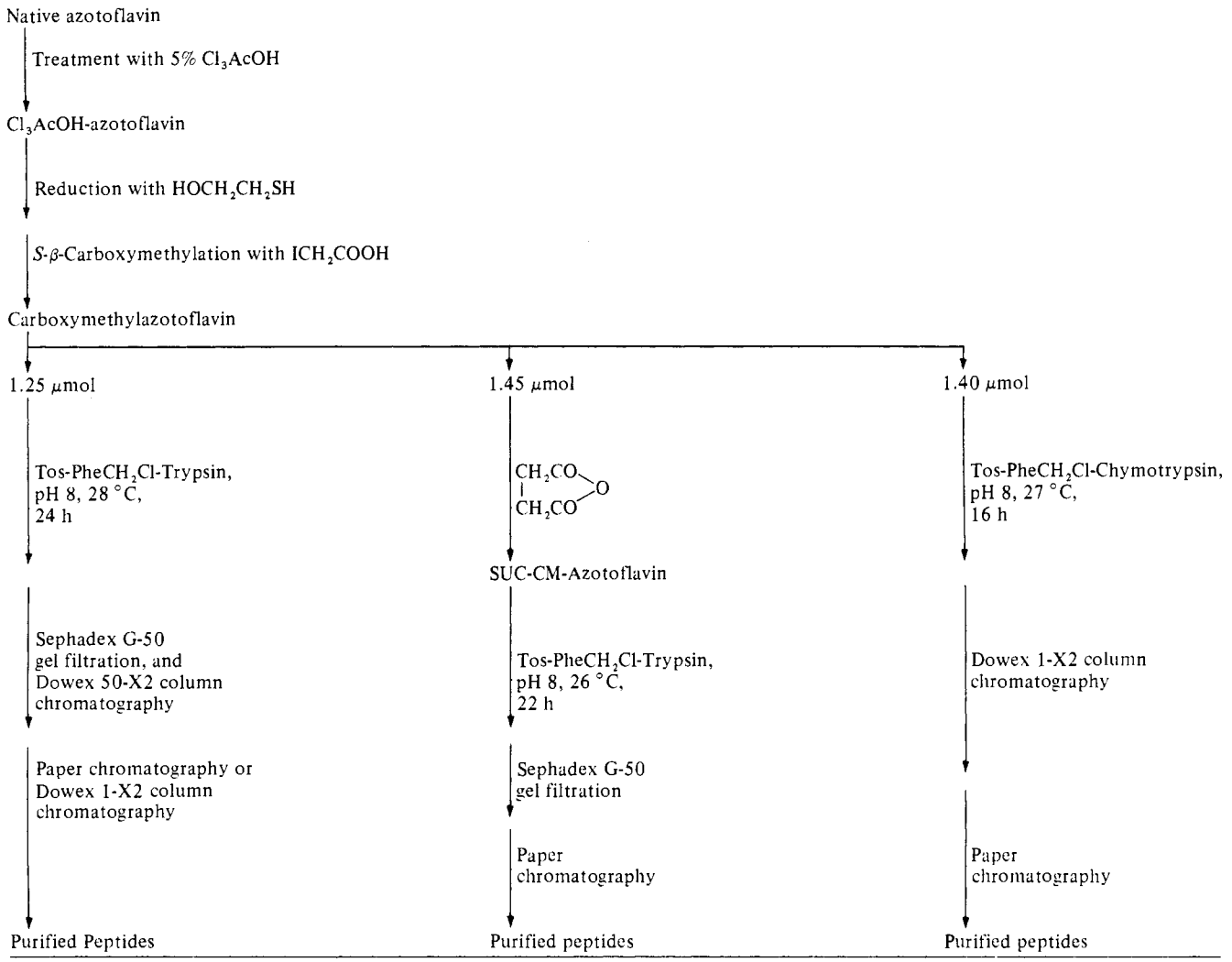
Purification of peptides was performed by some combination of the following methods: column chromatography on a cation-exchange resin, Dowex 50-X2 or on an anion-exchange resin, Dowex 1-X2, and paper chromatography in the solvent systems 1-butanol–pyridine–acetic acid–water (60:40:12:48, v/v); or pyridine–isoamyl alcohol–0.1 N ammonium hydroxide (60:30:50, v/v). Purification methods adopted for each peptide are shown in table of each experimental result.

Peptide Nomenclature. Tryptic peptides and chymotryptic peptides obtained from the hydrolysis of carboxymethylazotoflavin and tryptic peptides obtained from the hydrolysis of succinylated carboxymethylazotoflavin are designated by the symbols T, CT, and ST, respectively. Peptide fragments obtained from the thermolytic hydrolysis of peptide T10 are denoted by the symbol T10-Th. The numbers or letters after each symbol refer to the order in which the peptides are present in the protein or a particular peptide starting from their NH_2-terminal positions.

Results

Amino Acid Composition and End-Group Analyses of Azotoflavin. The amino acid composition of pure carboxymethylazotoflavin, which is reported in Table II, was determined by amino acid analyses after hydrolysis of the protein for 24, 48, and 72 h with 5.7 N HCl. Both the amino acid analyses and the sequence data (Table II) indicated that azotoflavin contained 179 amino acid residues. The amino acid

TABLE I: Strategy of Sequence Determinations of Azotoflavin.



composition of the Shethna flavoprotein from *Azotobacter vinelandii* (Table II) reported by Edmondson and Tollin (1971b), however, differed slightly from our results. Specifically, the Shethna flavoprotein was reported to contain one additional residue of lysine, arginine, aspartate, glycine, alanine, and tryptophan plus two additional residues of glutamate. The Shethna flavoprotein was reported to contain 187 amino acid residues, or 9 more residues than found in our preparation of azotoflavin.

End-group analyses of the carboxymethylazotoflavin included Sequencer analysis which yielded alanine in 100% yield at the first step of Edman degradation (see Sequence Determination section for details) and hydrazinolysis which released leucine in 97% yield. In these two experiments, no other amino acids were detected other than the end groups. Carboxypeptidase A digestion of the carboxymethyl-protein liberated serine (87%), glycine (77%), leucine (179%), and phenylalanine (75%) after 2 h of reaction, and serine (100%), glycine (92%), leucine (195%), and phenylalanine (90%) after 6 h of reaction. Thus, the NH_2- and COOH-terminal amino acids of the azotoflavin were shown to be H_2N-Ala ... (Phe, Gly,Leu,Ser)-Leu-COOH.

Isolation and Purification of Tryptic Peptides from Carboxymethylazotoflavin. For the initial separation, the tryptic digest of carboxymethylazotoflavin was separated on a Sephadex G-50 column and the chromatogram obtained is shown in Figure 1. Four peaks were detected by their absorbances at 280 nm. The first peak contained mainly peptide T10 together with some contaminants. This fraction was further purified by Dowex 1-X2 column chromatography and finally by paper chromatography (1-butanol-pyridine-acetic acid-water). The second peak contained four peptides, T9, T10b, T12, and T15. These peptides were further purified by paper chromatography (1-butanol-pyridine-acetic acid-water). The third peak contained six peptides, namely, T10a, T11, T16, T16b, T17, and T18. Paper chromatography of the peptide mixture in 1-butanol-pyridine-acetic acid-water separated three peptides, T16b, T17, and T18 in a pure form, while peptides T10a, T11, and T16 were coeluted from the paper. This peptide mixture (T10a, T11, and T16) was subjected to Dowex 1-X2 column chromatography and two peaks were detected. The first peak contained peptides T11 and T16, and the second peak contained pure peptide T10a. This peptide mixture was subjected to Dowex 50-X2 column chromatography and pure peptides T11 and T16 were obtained.

The fourth peak from the Sephadex G-50 column chromatography step (Figure 1) was shown to contain the eight peptides T1, T2, T3, T5, T6, T13, T14, and T16a, free lysine (T4 and T7), and free arginine (T8). The mixture containing these peptides and free amino acids was subjected to Dowex 50-X2 column chromatography and the elution pattern obtained is shown in Figure 2. Four peaks were observed. The first peak

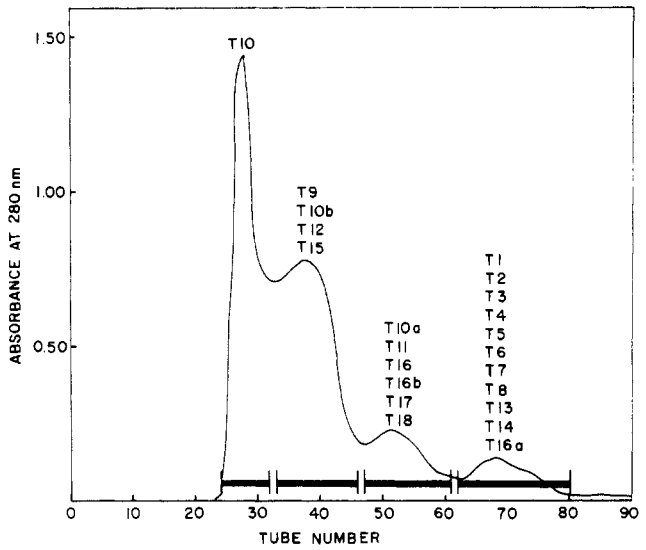


FIGURE 1: Exclusion-diffusion chromatography of the tryptic digest of carboxymethylazotoflavin on Sephadex G-50 gel. The tryptic digest of carboxymethylazotoflavin (about 1.25 μmol) was dissolved in 2.0 mL of 0.1 M ammonium hydroxide and immediately applied to a column of Sephadex G-50 (1.9 × 55 cm) equilibrated with the same buffer. The flow rate was 60 mL/h and 1.90-mL fractions were collected. Fractions under each peak which were pooled in Figures 1-5 are shown by solid bars.

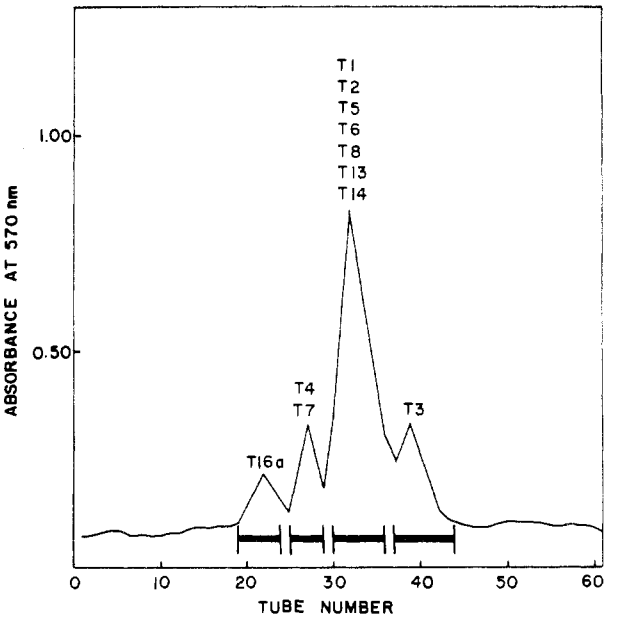


FIGURE 2: Cation-exchange column chromatography of the tryptic peptides from the fourth Sephadex G-50 fraction. The tryptic peptide fraction, which was fractionated on Sephadex G-50 gel, was next chromatographed on a column (1.0 × 20 cm) of Dowex 50-X2 resin. Linear gradient elution was carried out by mixing 100 mL of 0.2 M pyridinium-acetate buffer (pH 3.00) in the open mixing chamber with 100 mL of 2.0 M pyridinium-acetate buffer (pH 4.80) in the reservoir. The flow rate was 38 mL/h and fractions of 3.4 to 3.8 mL were collected. The peptides were detected by the ninhydrin reaction after alkaline hydrolysis (Crestfield et al., 1963).

TABLE II: Amino Acid Composition of Azotoflavin.

Amino acid	From present study[a]	From the sequence	Values[f] of Shethna flavoprotein
Lys	11.84 (12)	12	13
His	(0)	0	0
Arg	5.00 (5)	5	6
Asp	20.20 (20)	20[c]	21
Thr	7.80 (8)	8	8
Ser	14.60 (15)	15	15
Glu	21.00 (21)	21[d]	23
Pro	4.85 (5)	5	5
Gly	19.90 (20)	20	21
Ala	14.06 (14)	14	15
½-Cys	0.92 (1)	1	1
Val	10.00 (10)	10	10
Met	0.88 (1)	1	1
Ile	5.80 (6)	6	6
Leu	19.80 (20)	20	20
Tyr	5.00 (5)	5	5
Phe	12.80 (13)	13	13
Trp	2.74[b] (3)	3	4
Total res	179	179	187
Mol wt		19 990[e]	21 300

[a] Carboxymethylazotoflavin was hydrolyzed for 24, 48, and 72 h at 110 °C with 6 N HCl. The amino acid residues were calculated on the basis of an arginine content of 5.00 mol/mol of protein. Extrapolations were made for threonine and serine. Values for valine, isoleucine, and leucine were taken from 72-h hydrolysates. Values in parentheses indicate values rounded off to nearest whole number. [b] Determination by the procedure of Liu (1972). [c] Sum of 14 aspartic acids and 6 asparagines. [d] Sum of 16 glutamic acids and 5 glutamines. [e] Native protein including one FMN. [f] Taken from the reference of Edmondson and Tollin (1971).

contained peptides T16a and the pure form of the peptide was isolated by paper chromatography (1-butanol-pyridine-acetic acid-water). The second peak contained free lysine in 146% yield. It is most probable that those lysines came from residues 16 and 23 in the protein. The third peak contained six peptides (T1, T2, T5, T6, T13, and T14) and free arginine (T8). The mixture was subjected to paper chromatography (1-butanol-pyridine-acetic acid-water) and four peptides (T2, T5, T6, and T13) and free arginine (T8) were recovered in pure state. Peptides T1 and T14 could not however be separated in this solvent system. The fourth peak contained peptide T3 and it was purified by paper chromatography (1-butanol-pyridine-acetic acid-water).

The amino acid compositions and some properties of the tryptic peptides obtained from carboxymethylazotoflavin are summarized in Table III. A total of 18 tryptic peptides (T1 thru T18) and free lysine and arginine were isolated from the carboxymethyl derivative of the protein. The total of amino acid residues in the tryptic peptides was 179. This value is in excellent agreement with the determination of the amino acid composition of the protein. Four smaller trypsin digestion products, namely, peptides T10a, T10b, T16a and T16b, were useful in the sequence determination of the larger tryptic peptides T10 and T16.

Separation and Purification of Tryptic Peptides from Succinylated Carboxymethylazotoflavin. The tryptic digest of succinylated carboxymethylazotoflavin was chromatographed on a Sephadex G-50 column (1.9 × 57 cm) and the elution pattern obtained is shown in Figure 3. Three peaks were observed from their absorbances at 280 nm. The first peak contained two large peptides, ST4 and ST5. In order to obtain these peptides in pure form, paper chromatography using two kinds of solvent systems was necessary. The peptide mixture was first subjected to paper chromatography in the solvent system which consisted of pyridine-isoamyl alcohol-0.1 M ammonium hydroxide (60:30:50, v/v) and secondly in 1-butanol-pyridine-acetic acid-water (60:40:12:48, v/v). The second peak contained the four peptides ST1, ST2, ST3, and ST7. This peptide mixture was also purified by the combination

TABLE III: Amino Acid Compositions[a] and Properties of Tryptic Peptides from Carboxymethylazotoflavin.

Amino acid	T1 + T14[b]	T2	T3	T4 + T7[c]	T5	T6	T8	T9	T10[d]	T10a	T10b	T11	T12	T13	T15	T16[e]	T16a	T16b	T17	T18	Total res[f]
Lys	1.92 (2)	1.00 (1)		2.00 (2)	1.00 (1)	0.95 (1)			1.00 (1)		1.00 (1)	1.00 (1)	1.00 (1)		1.00 (1)	1.00 (1)		0.98 (1)			12
Arg			1.00 (1)				1.00 (1)	0.98 (1)						1.00 (1)					0.98 (1)		5
CM-Cys									0.78 (1)		0.82 (1)										1
Asp		1.09 (1)						4.86 (5)	3.00 (3)	1.02 (1)	2.06 (2)	1.05 (1)	3.03 (3)	0.98 (1)	2.03 (2)	2.94 (3)		3.05 (3)	1.02 (1)		20
Thr		0.99 (1)	0.98 (1)					0.95 (1)	1.85 (2)		1.92 (2)		0.87 (1)		0.95 (1)				0.94 (1)		8
Ser		0.87 (1)				0.80 (1)		0.88 (1)	3.69 (4)	0.86 (1)	2.74 (3)	0.82 (1)	0.83 (1)		3.65 (4)	0.84 (1)		0.85 (1)		0.86 (1)	15
Glu								1.03 (1)	8.76 (9)	2.98 (3)	5.95 (6)	1.01 (1)	3.04 (3)		3.04 (3)	0.96 (1)		1.03 (1)	1.00 (1)	1.98 (2)	21
Pro									2.70 (3)		2.84 (3)		0.98 (1)							0.93 (1)	5
Gly	1.00 (1)	2.96 (3)							4.05 (4)		4.04 (4)	2.00 (2)	4.02 (4)		2.98 (3)	1.92 (2)	1.00 (1)	1.02 (1)		1.02 (1)	20
Ala	2.00 (2)				1.00 (1)			1.00 (1)	1.96 (2)	2.00 (2)			2.00 (2)		1.00 (1)	1.00 (1)		1.00 (1)		3.98 (4)	14
Val					0.96 (1)			0.99 (1)	0.96 (1)	0.99 (1)			1.99 (2)		2.85 (3)	0.96 (1)	0.98 (1)			0.98 (1)	10
Met								0.85 (1)													1
Ile		1.03 (1)				1.00 (1)			0.97 (1)		1.00 (1)	0.94 (1)			0.99 (1)					1.00 (1)	6
Leu		1.01 (1)						1.02 (1)	5.93 (6)		5.95 (6)	0.98 (1)	4.94 (5)			3.02 (3)	1.02 (1)	1.98 (2)		3.02 (3)	20
Tyr									0.91 (1)	0.98 (1)			2.87 (3)		0.98 (1)						5
Phe		2.00 (2)						0.98 (1)	2.80 (3)	1.87 (2)	0.95 (1)	0.99 (1)	2.88 (3)		1.00 (1)	1.01 (1)	0.88 (1)			0.99 (1)	13
Trp[g]									0.86 (1)		0.87 (1)				0.89 (1)					0.95 (1)	3
Total res.	5	11	2	2	3	3	1	14	42	11	31	9	29	2	22	14	4	10	4	16	179
Yield (%)	48	46	45	73	84	60	48	68	59	25	31	64	47	63	79	28	36	45	75	55	
R_f in BPAW[h]	0.21	0.62	0.27	0.25	0.31	0.40	0.26	0.43	0.53	0.51	0.64	0.58	0.81	0.16	0.00	0.58	0.91	0.33	0.19	0.91	
Color react. with ninhydrin	Yellow to Violet	Violet	Yellow to Violet	Violet	Violet	Violet	Violet	Yellow to Violet	Violet	Violet	Violet	Violet	Violet	Violet	Violet	Violet	Violet	Violet	Yellow to Violet	Violet	
Ehrlich react.									Blue		Blue				Blue					Blue	
Purif. method[h]	Dowex 50 BPAW	Dowex 50 BPAW	Dowex 50 BPAW	Dowex 50 BPAW	Dowex 50 BPAW	Dowex 50 BPAW	Dowex 50 BPAW	BPAW	Dowex 1 BPAW	Dowex 1 BPAW	BPAW	Dowex 1 Dowex 50 BPAW	BPAW	Dowex 50 BPAW	BPAW	Dowex 1 Dowex 50 BPAW	Dowex 50 BPAW	BPAW	BPAW	BPAW	

[a] Results from 6 N HCl hydrolyses for 24 and 48 h. The numbers in parentheses refer to the assumed stoichiometric number of residues per molecule of pure peptide. [b] Mixture of two peptides, Ala-Lys (T1) and Gly-Ala-Lys (T14). See Results for experimental details. [c] Free lysine from the positions, 16 (T4) and 23 (T7), in the total sequence. [d] Sum of the peptides, T10a and T10b. [e] Sum of the peptides, T16a and T16b. [f] Sum of the peptides, T1–T18. [g] Determined by the procedure of Liu (1972). [h] The abbreviations used are: BPAW, ascending paper chromatography in the solvent system of 1-butanol–pyridine–acetic acid–water (60:40:12:48, v/v); Dowex 50, cation-exchange column chromatography on Dowex 50-X2 resin; Dowex 1, anion-exchange column chromatography on Dowex 1-X2 resin; res, residue; purif, purification; react., reaction.

TABLE IV: Amino Acid Compositions[a] and Properties of Tryptic Peptides from Succinylcarboxymethylazotoflavin.

Amino acid	ST1	ST2	ST3	ST4	ST5	ST6	ST7	Total res[d]
Lys	1.99 (2)	3.95 (4)		2.96 (3)	2.98 (3)			12
Arg	1.01 (1)	1.00 (1)	1.00 (1)	0.95 (1)	0.98 (1)			5
CM-Cys				0.84 (1)				1
Asp	1.00 (1)		5.01 (5)	7.97 (8)	6.00 (6)			20
Thr	1.88 (2)		0.97 (1)	2.88 (3)	1.96 (2)			8
Ser	0.84 (1)	0.85 (1)	0.86 (1)	5.78 (6)	4.74 (5)		0.84 (1)	15
Glu			0.95 (1)	12.71 (13)	4.86 (5)	0.98 (1)	0.99 (1)	21
Pro				3.85 (4)			0.98 (1)	5
Gly	2.89 (3)			9.80 (10)	5.96 (6)		0.95 (1)	20
Ala	0.96 (1)	0.98 (1)	0.99 (1)	4.00 (4)	3.02 (3)	3.00 (3)	1.00 (1)	14
Val		1.01 (1)	1.02 (1)	2.98 (3)	3.94 (4)	0.99 (1)		10
Met			0.84 (1)					1
Ile	0.94 (1)	0.99 (1)		2.02 (2)	1.00 (1)		1.00 (1)	6
Leu	1.00 (1)		1.03 (1)	11.80 (12)	3.01 (3)	1.02 (1)	2.02 (2)	20
Tyr				3.87 (4)	0.97 (1)			5
Phe	1.92 (2)		1.01 (1)	6.90 (7)	1.96 (2)		0.98 (1)	13
Trp[b]				0.82 (1)	0.85 (1)	0.86 (1)		3
Total res	15	9	14	82	43	7	9	179
Yield (%)	44	58	66	49	84	46	55	
R_f in BPAW[c]	0.70	0.20	0.54	0.80	0.00	0.76	0.88	
Color react. with ninhydrin		Violet	Violet	Violet	Yellow to Violet	Violet	Violet	
Sakaguchi react.	Brown	Brown	Brown	Brown	Brown			
Purif method[c]	BPAW PIN	BPAW PIN	BPAW PIN	BPAW PIN	BPAW PIN	BPAW	BPAW PIN	

[a] See footnote a in Table III. [b] See footnote g in Table III. [c] See footnote h in Table III. [d] Sum of the peptides, ST1–ST7.

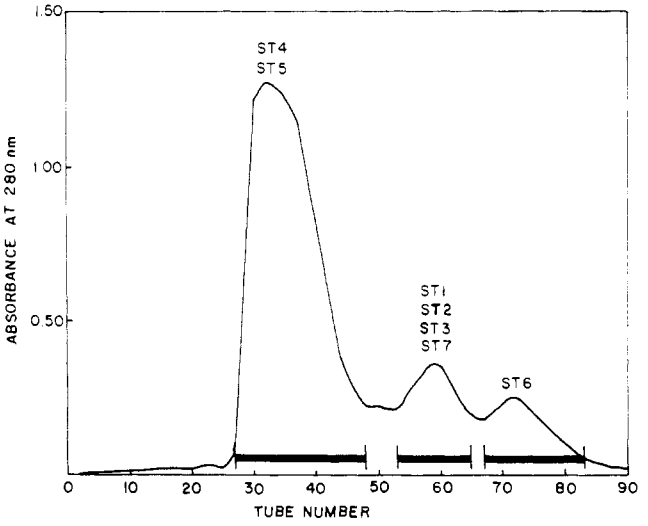


FIGURE 3: Sephadex G-50 exclusion-diffusion chromatography of the tryptic digest of succinylated carboxymethylazotoflavin. The tryptic digest of succinylated carboxymethylazotoflavin (about 1.45 μmol) was applied to a Sephadex G-50 column (1.9 × 57 cm) previously equilibrated with 0.1 M ammonium hydroxide which was also used for elution. The flow rate was 48 mL/h and 2.0-mL fractions were collected.

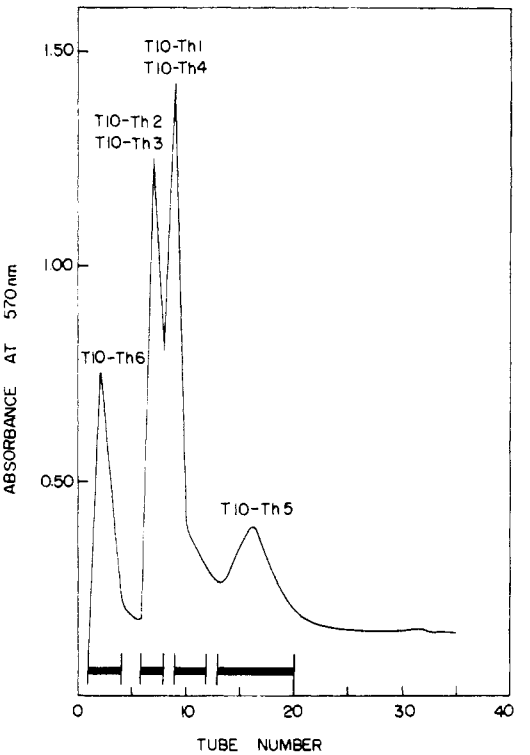


FIGURE 4: Anion-exchange column chromatography of the thermolytic peptides of peptide T10. The thermolytic digest of tryptic peptide T10 (about 0.5 μmol) was chromatographed on a Dowex 1-X2 column (0.35 × 7 cm). The peptides were eluted by linear gradient elution, carried out by mixing 25 mL of water in the open mixing chamber with 25 mL of 6.0 M acetic acid in the reservoir. The flow rate was 24 mL/h and fractions of 1.6 to 1.2 mL were collected. The peptides were assayed by the ninhydrin reaction after alkaline hydrolysis (Crestfield et al., 1963).

of paper chromatography using the two solvent systems just mentioned. The third peak contained mainly peptide ST6 together with some contaminants. It was purified by paper chromatography in 1-butanol–pyridine–acetic acid–water.

The amino acid composition and some properties of the tryptic peptides obtained from succinylated carboxymethylazotoflavin are summarized in Table IV. The total number of amino acid residues present in these seven tryptic peptides (ST1 to ST7) was 179.

Isolation and Purification of Thermolytic Peptides from Peptide T10. Shown in Figure 4 is the chromatographic pattern of the thermolytic digest of a tryptic peptide T10 on a Dowex 1-X2 column. Four peaks were detected by the ninhydrin procedure after alkaline hydrolysis. The first and fourth peaks contained the single peptides, T10-Th6 and T10-Th5,

TABLE V: Amino Acid Compositions[a] and Properties of Thermolytic Peptides from Peptide T10.

Amino acid	T10-Th1	T10-Th2	T10-Th3	T10-Th4	T10-Th5	T10-Th6	Total Res[d]
Lys						1.00 (1)	1
CM-Cys					0.88 (1)		1
Asp	1.00 (1)				2.00 (2)		3
Thr			1.89 (2)				2
Ser	0.87 (1)				2.84 (3)		4
Glu	0.97 (1)	2.00 (2)		2.00 (2)	4.06 (4)		9
Pro			0.85 (1)	0.95 (1)		0.96 (1)	3
Gly			1.00 (1)	2.89 (3)			4
Ala	1.00 (1)	0.98 (1)					2
Val	0.95 (1)						1
Ile			0.97 (1)				1
Leu			1.95 (2)	2.02 (2)	0.96 (1)	1.00 (1)	6
Tyr		0.96 (1)					1
Phe		1.95 (2)				0.98 (1)	3
Trp[b]					0.85 (1)		1
Total res.	5	6	7	8	12	4	42
Yield (%)	70	61	61	70	60	84	
R_f in BPAW[c]	0.36	0.44	0.56	0.53	0.30	0.61	
Color react. with ninhydrin	Violet	Violet	Violet	Violet	Violet	Violet	
Pauly react.		Pink					
Purif method[c]	BPAW	BPAW	BPAW	BPAW	BPAW	BPAW	

[a] See footnote a in Table III. [b] See footnote g in Table III. [c] See footnote h in Table III. [d] Sum of the peptides, T10-Th1–T10-Th6.

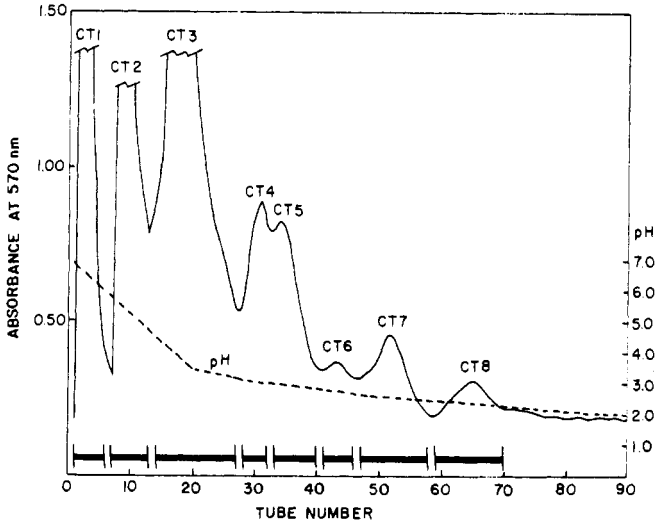


FIGURE 5: Anion-exchange column chromatography of the chymotryptic peptides of carboxymethylazotoflavin. The chymotryptic digest (about 1.40 μmol) was suspended in 1.0 mL of 0.1 M pyridine, dissolved by the addition of 10 drops of 20% pyridine, and then applied to a Dowex 1-X2 column (1.0 × 20 cm). Linear gradient elution was carried out by mixing 200 mL of 0.1 M pyridine in the open mixing chamber and 200 mL of 6.0 M acetic acid in the reservoir. The flow rate was 49 mL/h and fractions of 4.75 to 2.80 mL were collected. The peptide fractions were assayed by ninhydrin reaction after alkaline hydrolysis (Crestfield et al., 1963). The pH of the eluted fractions are shown by a dotted line.

respectively. The second peak contained two peptides, T10-Th2 and T10-Th3. The third peak also contained two peptides, T10-Th1 and T10-Th4. All of these thermolytic peptides (T10-Th1 thru T10-Th6) were further purified by paper chromatography in 1-butanol-pyridine-acetic acid-water (60:40:12:48, v/v). The amino acid composition and some pertinent properties of the thermolytic peptides from peptide T10 are summarized in Table V.

Fragmentation, Purification, and Sequence Studies of Chymotryptic Peptides from Carboxymethylazotoflavin. An outline of the experiments, which led to the isolation and sequence determinations of the key chymotryptic peptides, necessary for overlapping the carboxymethylazotoflavin will be described briefly in this section. The chymotryptic digest of carboxymethylazotoflavin (about 1.40 μmol) was directly applied to a Dowex 1-X2 column and the chromatogram obtained is shown in Figure 5. Ninhydrin assays were used to detect the eight fractions which were subsequently pooled separately. The first peak, CT1 was subjected to paper chromatography in pyridine-isoamyl alcohol-0.1 M ammonium hydroxide which separated six peptides. Two key peptides, CTb and CTc, were isolated from this fraction. The second peak, CT2, was separated by paper chromatography in 1-butanol-pyridine-acetic acid-water, and seven peptides as well as phenylalanine were present in the fraction. The third peak, CT3, contained seven peptides, and they were purified by paper chromatography in 1-butanol-pyridine-acetic acid-water. Two key peptides, CTa and CTe, were obtained from this fraction. The fourth peak, CT4 contained the essential peptide, CTd, together with some contaminants. This peptide was purified by paper chromatography in 1-butanol-pyridine-acetic acid-water. The fifth peak, CT5, contained two peptides, a glycyl peptide and a leucyl peptide which were separated by paper chromatography in 1-butanol-pyridine-acetic acid-water. No significant peptides were present in the sixth fraction CT6. The seventh peak, CT7, contained a single glycyl peptide which had the same amino acid composition as the glycyl peptide separated from the peak, CT5. However, manual Edman degradation of the glycyl peptide purified from the peak CT7 and CT5 showed that CT7 was a deaminated form of CT5 where a Gln to Glu difference at residue 5 was noted. The eighth fraction, CT8, contained a peptide which consisted of 25 amino acid residues, and it was purified by paper chromatography in 1-butanol-pyridine-acetic acid-water.

The amino acid compositions and sequences and some properties of the chymotryptic peptides are summarized in Table VI.

Sequence Investigations

Only the sequence data necessary to establish the total sequence of the azotoflavin are presented in this section, even

TABLE VI: Amino Acid Sequences and Properties of Chymotryptic Peptides from Carboxymethylazotoflavin.

Peak no.	Amino acid sequence[a]	Position in the total sequence	Peptide size	Yield (%)	R_f in solvent[a]	Color reaction with ninhydrin	Symbol for reconstruct. of the total sequence
CT1	Lys-Asp-Arg-Gly-Ala-Lys	118–123	6	14	0.16 (A)	Violet	
	Gly-Ser-Asn-Thr-Gly-Lys-Thr-Arg-Lys	8–16	9	19	0.28 (A)	Yellow to Violet	
	Gly-Ser-Asn-Thr-Gly-Lys-Thr-Arg-Lys-Val-Ala-Lys-Ser-Ile-Lys	8–22	15	19	0.38 (A)	Yellow to Violet	
	Lys-Asp-Arg-Gly-Ala-Lys-Ile-Val-Gly-Ser-Trp	118–128	11	30	0.57 (A)	Purple	CTc
	Ser-Gly-Lys-Thr-Val-Ala-Leu	87–93	7	45	0.65 (A)	Purple	CTb
	Ala-Lys-Ile-Gly-Leu-Phe	1–6	6	14	0.83 (A)	Violet	
CT2	Asn-Val-Asn-Arg-Val-Ser-Ala-Glu-Asp-Phe	35–44	10	24	0.35 (B)	Violet	
	Ala-Gln-Tyr	45–47	3	70	0.46 (B)	Violet	
	Ser-Leu	178–179	2	69	0.60 (B)	Violet	
	Phe	7 and 94	1	69	0.60 (B)	Violet	
	Ala-Leu	150–151	2	12	0.68 (B)	Violet	
	Val-Gly-Leu	147–149	3	24	0.76 (B)	Violet	
	Gln-Phe-Leu-Ile-Leu	48–52	5	14	0.84 (B)	Violet	
	Val-Gly-Leu-Ala-Leu	147–151	5	20	0.91 (B)	Violet	
CT3	Lys-Arg-Phe-Asp-Asp-Glu-Thr-Met-Ser-Asp-Ala-Leu	23–24	12	22	0.26 (B)	Violet	
	Asp-Leu-Asp-Asn-Glu-Ser-Gly-Lys-Thr-Asp-Glu-Arg-Val-Ala-Ala-Trp-Leu	152–168	17	18	0.35 (B)	Violet	
	Ala-Leu-Asp-Leu-Asp-Asn-Glu-Ser-Gly-Lys-Thr-Asp-Glu-Arg-Val-Ala-Ala-Trp-Leu	150–168	19	55	0.55 (B)	Violet	CTe
	Leu-Pro-Lys-Ile-Glu-Gly-Leu-Asp-Phe	78–86	9	34	0.65 (B)	Violet	CTa
	Ala-Gln-Ile-Ala-Pro-Glu-Phe-Gly-Leu	169–177	9	24	0.72 (B)	Violet	
	Ser-Phe-Phe	115–117	3	48	0.79 (B)	Yellow to Violet	
	Leu-Ala-Gln-Ile-Ala-Pro-Glu-Phe-Gly-Leu	168–177	10	12	0.87 (B)	Violet	
CT4	Ser-Thr-Asp-Gly-Tyr-Glu-Phe-Glu-Ser-Ser-Glu-Ala-Val-Val-Asp-Gly-Lys-Phe	129–146	18	48	0.39 (B)	Violet	CTd
CT5	Gly-Leu-Gly-Asp-Gln-Val-Gly-Tyr-Pro-Glu-Asn-Tyr	95–106	12	24	0.59 (B)	Yellow to Violet	
	Leu-Asp-Ala-Leu-Gly-Glu-Leu-Tyr	107–114	8	38	0.83 (B)	Violet	
CT7	Gly-Leu-Gly-Asp-Glu-Val-Gly-Tyr-Pro-Glu-Asp-Tyr	95–106	12	32	0.66 (B)	Yellow to Violet	
CT8	Gly-Thr-Pro-Thr-Leu-Gly-Glu-Gly-Glu-Leu-Pro-Gly-Leu-Ser-Ser-Asp-CMC-Glu-Asn-Glu-Ser-Trp-Glu-Glu-Phe	53–77	25	24	0.37 (B)	Yellow to Violet	

[a] The abbreviations used are: CMC, *S*-β-carboxymethylcysteine; A and B, paper chromatography in the solvent systems, pyridine–isoamyl alcohol–0.1 M ammonium hydroxide (60:30:50, v/v); and 1-butanol–pyridine–acetic acid–water (60:40:12:48, v/v), respectively.

though additional sequence studies were performed on other peptides. In the following sequence investigations, the abbreviations used for the analytical methods for the determination of Pth-amino acids are: (1) GLC, gas–liquid chromatography of the Pth-amino acid; (2) TLC, thin-layer chromatography of the Pth-amino acid; and (3) AAA, amino acid analysis of the 6 N HCl hydrolysate (20 h at 150 °C) of the Pth-amino acid. Also, the three-letter abbreviation "CMC" is used for the *S*-β-carboxymethylcysteine residue in this section.

Protein Sequenator Results of Carboxymethylazotoflavin (Residues 1–53). About 200 nmol of the carboxymethylazotoflavin was analyzed twice in the Beckman-Spinco Protein Sequencer. The protein double-cleavage program and 1.0 M Quadrol as the coupling buffer were used for sequencing. The amino acid sequence of the first 53 residues from the NH_2-terminal position of the protein was determined and the sequence results are summarized in Table VII. The average repetitive yield was calculated to be 95.4%.

Sequenator Results of Peptide T10 (Residues 39–68). Sequence analyses were performed on both the peptide T10 (200 nmol) and the 4-sulfophenylthiocarbamyl derivative of the peptide T10 (300 nmol). The sequencing was carried out in the Sequencer utilizing the protein single-cleavage program. From the combination of these experimental results, it was possible to determine the first 30 residues from the NH_2-terminal end of the peptide. The experimental results obtained are summarized in Table VIII. Hydrazinolysis of the intact peptide released lysine in 54% yield.

Peptide T10-Th5: Leu-Ser-Ser-Asp-CMC-Glu-Asn-Glu-Ser-Trp-Glu-Glu (Residues 65–76). Manual Edman degradation established the sequence of this peptide. The yields of the Pth-amino acids and methods of analyses were as follows: step 1, Pth-Leu, 100% (GLC, AAA); step 2, Pth-Ser, 80% (GLC, TLC); step 3, Pth-Ser, 68% (GLC, TLC); step 4, Pth-Asp, 60% (AAA, TLC); step 5, Pth-CMC, 49% (GLC, TLC); step 6, Pth-Glu, 40% (AAA, TLC); step 7, Pth-Asn, 32% (AAA, TLC); step 8, Pth-Glu, 26% (AAA, TLC); step 9, Pth-Ser, 20% (GLC, TLC); step 10, Pth-Trp, 15% (GLC, TLC); and step 11, Pth-Glu, 13% (AAA, TLC). After the eleventh step of Edman degradation, free glutamic acid was present in 12% yield when the residue was analyzed by amino acid analysis without an acid-hydrolysis step. Hydrazinolysis of the intact peptide released glutamic acid in 86% yield.

Peptide T10-Th6: Phe-Leu-Pro-Lys (Residues 77–80). Three steps of manual Edman degradation yielded the following results: step 1, Pth-Phe, 95% (GLC, AAA); step 2, Pth-Leu, 80% (GLC, AAA); and step 3, Pth-Pro, 56% (GLC, AAA). When the residual sample after the third step was analyzed in the amino acid analyzer without acid hydrolysis, ε-phenylthiocarbamyllysine was found in 50% yield.

TABLE VII: Results of the Automatic Sequenator Analysis of Carboxymethylazotoflavin.

Step no.	Sequence[a]	% Yield	Methods of Analysis[b]	
1	Ala	100	GLC	AAA
2	Lys	64	AAA	
3	Ile	95	GLC	AAA
4	Gly	91	GLC	AAA
5	Leu	88	GLC	AAA
6	Phe	85	GLC	AAA
7	Phe	83	GLC	AAA
8	Gly	80	GLC	AAA
9	Ser	76	GLC	TLC
10	Asp	75	AAA	TLC
11	Thr	72	GLC	TLC
12	Gly	70	GLC	AAA
13	Lys	44	AAA	
14	Thr	64	GLC	TLC
15	Arg	20	AAA	
16	Lys	40	AAA	
17	Val	59	GLC	AAA
18	Ala	57	GLC	AAA
19	Lys	33	AAA	
20	Ser	52	GLC	TLC
21	Ile	51	GLC	AAA
22	Lys	30	AAA	
23	Lys	28	AAA	
24	Arg	14	AAA	
25	Phe	44	GLC	AAA
26	Asp	42	AAA	TLC
127	Asp	41	AAA	TLC
28	Glu	39	AAA	TLC
29	Thr	36	GLC	TLC
30	Met	35	GLC	AAA
31	Ser	33	GLC	TLC
32	Asp	33	AAA	TLC
33	Ala	32	GLC	AAA
34	Leu	31	GLC	AAA
35	Asp	29	AAA	TLC
36	Val	28	GLC	AAA
37	Asp	26	AAA	TLC
38	Arg	6	AAA	
39	Val	24	GLC	AAA
40	Ser	22	GLC	TLC
41	Ala	22	GLC	AAA
42	Glu	20	AAA	TLC
43	Asp	19	AAA	TLC
44	Phe	18	GLC	AAA
45	Ala	17	GLC	AAA
46	Glu	16	AAA	TLC
47	Tyr	14	GLC	AAA
48	Glu	14	AAA	TLC
49	Phe	13	GLC	AAA
50	Leu	12	GLC	AAA
51	Ile	11	GLC	AAA
52	Leu	10	GLC	AAA
53	Gly	9	GLC	AAA

[a] Carboxymethylazotoflavin (200 nmol) was analyzed in the Beckman-Spinco Model 890 Protein Sequencer. [b] Identification methods used for analyses of the Pth-amino acid released at each step of Edman degradation were gas liquid chromatography (GLC), and/or thin-layer chromatography (TLC), and/or amino acid analysis (AAA) of the 6 N HCl hydrolysate of the Pth-amino acid. See the text for experimental details.

TABLE VIII: Sequenator Analysis of Peptide T10.

Step no.	Sequence[a]	% Yield	Methods of Analysis[b]	
1	Val	100	GLC	AAA
2	Ser	93	GLC	TLC
3	Ala	90	GLC	AAA
4	Glu	85	AAA	TLC
5	Asp	81	AAA	TLC
6	Phe	74	GLC	AAA
7	Ala	70	GLC	AAA
8	Glu	65	AAA	TLC
9	Tyr	58	GLC	AAA
10	Glu	56	AAA	TLC
11	Phe	51	GLC	AAA
12	Leu	48	GLC	AAA
13	Ile	44	GLC	AAA
14	Leu	42	GLC	AAA
15	Gly	38	GLC	AAA
16	Thr	35	GLC	TLC
17	Pro	31	GLC	AAA
18	Thr	29	GLC	TLC
19	Leu	27	GLC	AAA
20	Gly	24	GLC	AAA
21	Glu	23	AAA	TLC
22	Gly	20	GLC	AAA
23	Glu	18	AAA	TLC
24	Leu	16	GLC	AAA
25	Pro	14	GLC	AAA
26	Gly	13	GLC	AAA
27	Leu	12	GLC	AAA
28	Ser	10	GLC	
29	Ser	8	GLC	
30	Asp	8	AAA	TLC

[a] Both the peptide T10 (200 nmol) and the 4-sulfophenylthiocarbamyl derivative of the peptide T10 (300 nmol) were analyzed in the Beckman-Spinco Model 890 Protein/Peptide Sequencer. See the text for experimental details. [b] See footnote b in Table VII.

Peptide CTa: Leu-Pro-Lys-Ile-Glu-Gly-Leu-Asp-Phe (Residues 78–86). The sequence results of eight steps of Edman degradation were as follows: step 1, Pth-Leu, 100% (GLC, AAA); step 2, Pth-Pro, 88% (GLC, AAA); step 3, Pth-Lys, 50% (AAA); step 4, Pth-Ile, 62% (GLC, AAA); step 5, Pth-Glu, 48% (AAA, TLC); step 6, Pth-Gly, 39% (GLC, AAA); step 7, Pth-Leu, 32% (GLC, AAA); and step 8, Pth-Asp, 24% (AAA, TLC). After the eighth step of Edman degradation, free phenylalanine was present in the residual sample in 18% yield as determined by amino acid analysis of the sample. Hydrazinolysis of the intact peptide released phenylalanine in 74% yield.

Peptide T11: Ile-Glu-Gly-Leu-Asp-Phe-Ser-Gly-Lys (Residues 81–89). Eight steps of manual Edman degradation of this peptide yielded the following products at each step: step 1, Pth-Ile, 100% (GLC, AAA); step 2, Pth-Glu, 82% (AAA, TLC); step 3, Pth-Gly, 65% (GLC, AAA); step 4, Pth-Leu, 56% (GLC, AAA); step 5, Pth-Asp, 44% (AAA, TLC); step 6, Pth-Phe, 37% (GLC, AAA); step 7, Pth-Ser, 28% (GLC, TLC); and step 8, Pth-Gly, 25% (GLC, AAA). The residual sample after the eighth step of Edman degradation contained ϵ-phenylthiocarbamyllysine in 20% yield. Carboxypeptidase B digestion of the intact peptide liberated lysine in 100% yield.

Peptide CTb: Ser-Gly-Lys-Thr-Val-Ala-Leu (Residues 87–93). Six steps of manual Edman degradation established the amino acid sequence of this peptide to be: step 1, Pth-Ser, 90% (GLC, TLC); step 2, Pth-Gly, 78% (GLC, AAA); step 3, Pth-Lys, 52% (AAA); step 4, Pth-Thr, 50% (GLC, TLC); step 5, Pth-Val, 44% (GLC, AAA); and step 6, Pth-Ala, 36% (GLC, AAA). The residual sample after the sixth step of Edman degradation was applied directly on the amino acid analyzer and free leucine was present in 30% yield. Hydrazinolysis of the intact peptide released leucine in 80% yield.

Modified Sequenator Results of Peptide T12 (Residues

TABLE IX: Modified Sequenator Analysis of Peptide T12.

Step no.	Sequence[a]	% Yield	Methods of Analysis[b]	
1	Thr	96	GLC	TLC
2	Val	94	GLC	AAA
3	Ala	88	GLC	AAA
4	Leu	83	GLC	AAA
5	Phe	75	GLC	AAA
6	Gly	67	GLC	AAA
7	Leu	62	GLC	AAA
8	Gly	55	GLC	AAA
9	Asp	50	AAA	TLC
10	Glu	47	AAA	TLC
11	Val	44	GLC	AAA
12	Gly	40	GLC	AAA
13	Tyr	35	GLC	AAA
14	Pro	32	GLC	AAA
15	Glu	30	AAA	TLC
16	Asp	27	AAA	TLC
17	Tyr	24	GLC	AAA
18	Leu	23	GLC	AAA
19	Asp	21	AAA	TLC
20	Ala	20	GLC	AAA
21	Leu	19	GLC	AAA
22	Gly	16	GLC	AAA
23	Glu	15	AAA	TLC
24	Leu	15	GLC	AAA
25	Tyr	13	GLC	AAA
26	Ser	11	GLC	TLC
27	Phe	10	GLC	AAA
28	Phe	10	GLC	AAA
28R[c]	Lys	8	AAA	

[a] Both the peptide T12 (200 nmol) and the 4-sulfophenylthiocarbamylated peptide T12 (250 nmol) were initially analyzed in the Beckman-Spinco Model 890 Protein/Peptide Sequencer. In the latter analyses, the residual peptide after step 16 was removed from the cup and manual Edman degradation was subsequently carried out from steps 17 to 28. See the text for experimental details. [b] See footnote b in Table VII. [c] The residual sample after step 28 was hydrolyzed in 6 N HCl at 150 °C for 20 h and analyzed in the amino acid analyzer.

90–118). In order to quantitatively complete the sequence of this peptide, sequence analyses were performed on both the 4-sulfophenylthiocarbamyl derivative of the peptide (250 nmol) and the underivatized peptide (200 nmol). The sequencing was carried out in the Sequencer using the protein single-cleavage program. When the peptide T12 was first run in the Sequencer, it was observed that the yield of the Pth-tyrosine dropped greatly after step 16 in the Edman degradation. In the second analysis in the Sequencer, the 4-sulfophenylthiocarbamyl derivative of the peptide T12 was run, and, after step 16, the residual peptide was removed from the cup with 20% pyridine and transferred to a test tube (Tanaka et al., 1975). Thus, manual Edman degradation was subsequently carried out from steps 17 to 28 to complete the sequence of the peptide. Typical sequence results of the peptide T12 are summarized in Table IX. Carboxypeptidase B digestion of the intact peptide liberated lysine in 100% yield and hydrazinolysis of the residual peptide after the digestion released phenylalanine in 78% yield.

Peptide CTc: Lys-Asp-Arg-Gly-Ala-Lys-Ile-Val-Gly-Ser-Trp (*Residues* 118–128). Ten steps of manual Edman degradation of this peptide yielded at each step the following Pth-amino acids: step 1, Pth-Lys, 92% (AAA); step 2, Pth-Asp, 80% (AAA, TLC); step 3, Pth-Arg, 16% (AAA); step 4, Pth-Gly, 56% (GLC, AAA); step 5, Pth-Ala, 50% (GLC, AAA); step 6, Pth-Lys, 32% (AAA); step 7, Pth-Ile, 35% (GLC, AAA); step 8, Pth-Val, 30% (GLC, AAA); step 9, Pth-Gly, 24% (GLC, AAA); and step 10, Pth-Ser, 20% (GLC, TLC). The residual sample after the tenth step of Edman degradation was applied directly on the amino acid analyzer and tryptophan was present in 15% yield. Hydrazinolysis of the intact peptide liberated tryptophan in 75% yield.

A Mixture of Two Peptides: T1, Ala-Lys (*Residues 1–2*), *and T14, Gly-Ala-Lys* (*Residues 121–123*). One step of manual Edman degradation of this peptide mixture yielded Pth-Ala in 100% yield and Pth-Gly in 96% yield which were analyzed by GLC and AAA. When one-fifth of the residual sample after the first step of Edman degradation was analyzed directly in the amino acid analyzer without 6 N HCl hydrolysis, free ϵ-phenylthiocarbamyllysine was detected in 89% yield. The subsequent Edman degradation on the four-fifths remainder of the sample after the first step of Edman degradation yielded Pth-Ala in 85% yield (by GLC, AAA), and the residual sample after this step of Edman degradation yielded ϵ-phenylthiocarbamyllysine in 72% yield. From the above-mentioned Edman degradation results as well as from the amino acid composition data (Table III) it was concluded that this peptide mixture contained an equimolar ratio of the two peptides, Ala-Lys (peptide T1) and Gly-Ala-Lys (peptide T14). These peptides were derived from the positions 1–2 and from the positions 121–123, respectively.

Peptide T15: Ile-Val-Gly-Ser-Trp-Ser-Thr-Asp-Gly-Tyr-Glu-Phe-Glu-Ser-Ser-Glu-Ala-Val-Val-Asp-Gly-Lys (*Residues 124–145*). Twenty-one steps of manual Edman degradation of this peptide yielded the following sequence results: step 1, Pth-Ile, 100% (GLC, AAA); step 2, Pth-Val, 92% (GLC, AAA); step 3, Pth-Gly, 82% (GLC, AAA); step 4, Pth-Ser, 74% (GLC, TLC); step 5, Pth-Trp, 65% (GLC, TLC); step 6, Pth-Ser, 60% (GLC, TLC); step 7, Pth-Thr, 58% (GLC, TLC); step 8, Pth-Asp, 54% (AAA, TLC); step 9, Pth-Gly, 49% (GLC, AAA); step 10, Pth-Tyr, 40% (GLC, AAA); step 11, Pth-Glu, 38% (AAA, TLC); step 12, Pth-Phe, 33% (GLC, AAA); step 13, Pth-Glu, 30% (AAA, TLC); step 14, Pth-Ser, 26% (GLC, TLC); step 15, Pth-Ser, 23% (GLC, TLC); step 16, Pth-Glu, 22% (AAA, TLC); step 17, Pth-Ala, 20% (GLC, AAA); step 18, Pth-Val, 18% (GLC, AAA); step 19, Pth-Val, 17% (GLC, AAA); step 20, Pth-Asp, 14% (AAA, TLC); and step 21, Pth-Gly, 12% (GLC, AAA). The amino acid analysis of the residual sample after the twenty-first step of Edman degradation yielded free ϵ-phenylthiocarbamyllysine in 9% yield. Carboxypeptidase B digestion plus hydrazinolysis of the intact peptide liberated lysine and glycine in 100 and 73% yields, respectively.

Peptide CTd: Ser-Thr-Asp-Gly-Tyr-Glu-Phe-Glu-Ser-Ser-Glu-Ala-Val-Val-Asp-Gly-Lys-Phe (*Residues 129–146*). Seventeen steps of manual Edman degradation of the peptide CTd yielded the following results at each of the steps: step 1, Pth-Ser, 95% (GLC, TLC); step 2, Pth-Thr, 86% (GLC, TLC); step 3, Pth-Asp, 80% (AAA, TLC); step 4, Pth-Gly, 70% (GLC, AAA); step 5, Pth-Tyr, 62% (GLC, AAA); step 6, Pth-Glu, 58% (AAA, TLC); step 7, Pth-Phe, 51% (GLC, AAA); step 8, Pth-Glu, 47% (AAA, TLC); step 9, Pth-Ser, 40% (GLC, TLC); step 10, Pth-Ser, 35% (GLC, TLC); step 11, Pth-Glu, 32% (AAA, TLC); step 12, Pth-Ala, 30% (GLC, AAA); step 13, Pth-Val, 26% (GLC, AAA); step 14, Pth-Val, 23% (GLC, AAA); step 15, Pth-Asp, 19% (AAA, TLC); step 16, Pth-Gly, 16% (GLC, AAA); and step 17, Pth-Lys, 11% (AAA). When the residual sample after the seventeenth step of Edman degradation was analyzed directly in the amino acid analyzer, free phenylalanine was present in 8% yield. Hydrazinolysis of the intact peptide released phenylalanine in 82% yield. Hydrolysis of the intact peptide by carboxypeptidase A

liberated phenylalanine in 81% yield after 1 h and phenylalanine in 100% yield after 6 h. Carboxypeptidase B digestion of the residual sample after the carboxypeptidase A hydrolysis released lysine in 94% yield after 1 h in addition to phenylalanine. When the residual sample after the above-mentioned carboxypeptidase A–B digestion experiment was subjected to hydrazinolysis, glycine was obtained in 91% yield in addition to the phenylalanine and lysine. Thus, the COOH-terminal sequence of the peptide CTd was confirmed to be Gly-Lys-Phe-COOH.

Peptide T16: Phe-Val-Gly-Leu-Ala-Leu-Asp-Leu-Asp-Asn-Gln-Ser-Gly-Lys (Residues 146–159). Thirteen steps of manual Edman degradation were carried out on this peptide and the yields of Pth-amino acids at each step were as follows: step 1, Pth-Phe, 96% (GLC, AAA); step 2, Pth-Val, 88% (GLC, AAA); step 3, Pth-Gly, 72% (GLC, AAA); step 4, Pth-Leu, 60% (GLC, AAA); step 5, Pth-Ala, 50% (GLC, AAA); step 6, Pth-Leu, 44% (GLC, AAA); step 7, Pth-Asp, 35% (AAA, TLC); step 8, Pth-Leu, 32% (GLC, AAA); step 9, Pth-Asp, 28% (AAA, TLC); step 10, Pth-Asn, 25% (AAA, TLC); step 11, Pth-Gln, 21% (AAA, TLC); step 12, Pth-Ser, 16% (GLC, TLC); and step 13, Pth-Gly, 14% (GLC, AAA). The residual sample after the thirteenth step of Edman degradation yielded ε-phenylthiocarbamyllysine in 10% yield when the sample was analyzed in the amino acid analyzer without 6 N HCl hydrolysis. Carboxypeptidase B digestion and then hydrazinolysis of the intact peptide yielded lysine in 100% and glycine in 77% yields, respectively.

Peptide CTe: Ala-Leu-Asp-Leu-Asp-Asn-Gln-Ser-Gly-Lys-Thr-Asp-Glu-Arg-Val-Ala-Ala-Trp-Leu (Residues 150–168). Eighteen steps of manual Edman degradation of this peptide yielded at each step: step 1, Pth-Ala, 100% (GLC, AAA); step 2, Pth-Leu, 93% (GLC, AAA); step 3, Pth-Asp, 85% (AAA, TLC); step 4, Pth-Leu, 76% (GLC, AAA); step 5, Pth-Asp, 70% (AAA, TLC); step 6, Pth-Asn, 62% (AAA, TLC); step 7, Pth-Gln, 56% (AAA, TLC); step 8, Pth-Ser, 47% (GLC, TLC); step 9, Pth-Gly, 42% (GLC, AAA); step 10, Pth-Lys, 25% (AAA); step 11, Pth-Thr, 33% (GLC, TLC); step 12, Pth-Asp, 30% (AAA, TLC); step 13, Pth-Glu, 26% (AAA, TLC); step 14, Pth-Arg, 8% (AAA); step 15, Pth-Val, 21% (GLC, AAA); step 16, Pth-Ala, 18% (GLC, AAA); step 17, Pth-Ala, 17% (GLC, AAA); and step 18, Pth-Trp, 12% (GLC, TLC). After the eighteenth step of Edman degradation, free leucine was recovered in 11% yield when the residual sample was subjected to amino acid analysis without 6 N HCl hydrolysis. Hydrazinolysis of the intact peptide released leucine in 76% yield.

Peptide T18: Val-Ala-Ala-Trp-Leu-Ala-Gln-Ile-Ala-Pro-Glu-Phe-Gly-Leu-Ser-Leu (Residues 164–179). Fifteen steps of manual Edman degradation established the amino acid sequence of the peptide T18. The yields and determination methods of the Pth-amino acids at each step were as follows: step 1, Pth-Val, 100% (GLC, AAA); step 2, Pth-Ala, 94% (GLC, AAA); step 3, Pth-Ala, 87% (GLC, AAA); step 4, Pth-Trp, 73% (GLC, TLC); step 5, Pth-Leu, 70% (GLC, AAA); step 6, Pth-Ala, 65% (GLC, AAA); step 7, Pth-Gln, 56% (AAA, TLC); step 8, Pth-Ile, 50% (GLC, AAA); step 9, Pth-Ala, 45% (GLC, AAA); step 10, Pth-Pro, 38% (GLC, AAA); step 11, Pth-Glu, 35% (AAA, TLC); step 12, Pth-Phe, 30% (GLC, AAA); step 13, Pth-Gly, 26% (GLC, AAA); step 14, Pth-Leu, 23% (GLC, AAA); and step 15, Pth-Ser, 18% (GLC, TLC). When the residual sample after the fifteenth step was analyzed in the amino acid analyzer without acid hydrolysis, free leucine was found in 15% yield. Hydrazinolysis of the peptide T18 liberated leucine in 81% yield.

Correction of Residue 156. In our preliminary report (Tanaka et al., 1975) it was reported that residue 156 was glutamic acid. This was due to a typographical error and should be corrected to glutamine.

Complete Sequence. The NH_2- and COOH-terminal amino acids of the azotoflavin were determined to be alanine and leucine, respectively, by Sequencer analysis and by hydrazinolysis. The automatic sequenator analysis established the sequence of the first 53 residues from the NH_2-terminal position of azotoflavin. The Sequencer results made it possible to order the tryptic peptides T1 thru T10. Peptide CTa overlapped peptides T10 and T11. Peptide CTb overlapped peptides T11 and T12. Peptide CTc overlapped peptides T12, T13, T14 and T15 in that order. Peptide CTd overlapped peptides T15 and T16. Finally, peptide CTe overlapped peptides T16, T17, and T18 in that order. The above-mentioned results were confirmed by the sequence studies on the tryptic peptides obtained from succinylated carboxymethylazotoflavin. The sequence studies of the large peptide T10 which consisted of 42 amino acid residues were completed by combination of the Sequencer analysis and the sequence analyses of the thermolytic peptides of the peptide T10. Reconstruction of the complete amino acid sequence of the azotoflavin, which was derived from Protein Sequencer analysis and sequence studies of tryptic and chymotryptic peptides of the carboxymethyl derivative of the protein as well as of tryptic peptides of succinylated carboxymethylprotein, is summarized in Figure 6.

Discussion

The biological activity of *Azotobacter* flavodoxin is not well understood (Benemann et al., 1971; Yoch, 1972); this flavoprotein, nevertheless, is of considerable interest because of the rather exceptional stability of its semiquinone form to both reduction by strong reducing agents and oxidation by O_2. An extensive study by Tollin and associates has been directed toward an understanding of these unusual oxidation-reduction properties of *Azotobacter* flavodoxin (Edmondson and Tollin, 1971a, 1971c; Barman and Tollin, 1972).

Because all flavodoxins have the same prosthetic group, any differences in oxidation-reduction behavior are an indication of the influence of the polypeptide chain. In this regard, MacKnight et al. (1974) have determined the amino-terminal sequence (approximately 45 residues) of *A. vinelandii* flavodoxin and compared it to those of five flavodoxins: *Peptostreptococcus elsdenii* (Tanaka et al., 1973; 1974b), *Clostridium MP* (Tanaka et al., 1974a); *Clostridium pasteurianum* (Tanaka et al., 1971; Fox et al., 1972) and *Desulfovibrio vulgaris* (Dubourdieu et al., 1973) and *Rhodospirillum rubrum* (MacKnight et al., 1974). While a significant amino acid homology was shown for the *Azotobacter* flavodoxin in the region of the FMN-binding site, there was reported to be no correlation between *Azotobacter* flavodoxin and the other flavodoxins outside of this region (MacKnight et al., 1974). At the time the report of MacKnight appeared (1974), sequence studies of the *A. vinelandii* flavodoxin were in progress in our laboratory. Interest in the primary sequence of our flavodoxin was maintained, however, when it was shown (Yoch, 1975) that the oxidation-reduction properties of the flavodoxin from *Azotobacter* strain OP (Berkeley), whose sequence we are now reporting, were quite different from those of other strains of *Azotobacter*. For example, the flavodoxin from strain OP is reduced to a mixture of about half semiquinone and half hydroquinone at pH 7, whereas other *Azotobacter* flavodoxins are not reduced beyond the semiquinone form at neutral pH. Another significant difference between the flavodoxins of these strains is the oxidation-reduction potential of the oxidized semiquinone couple. Barman and Tollin (1972) reported that

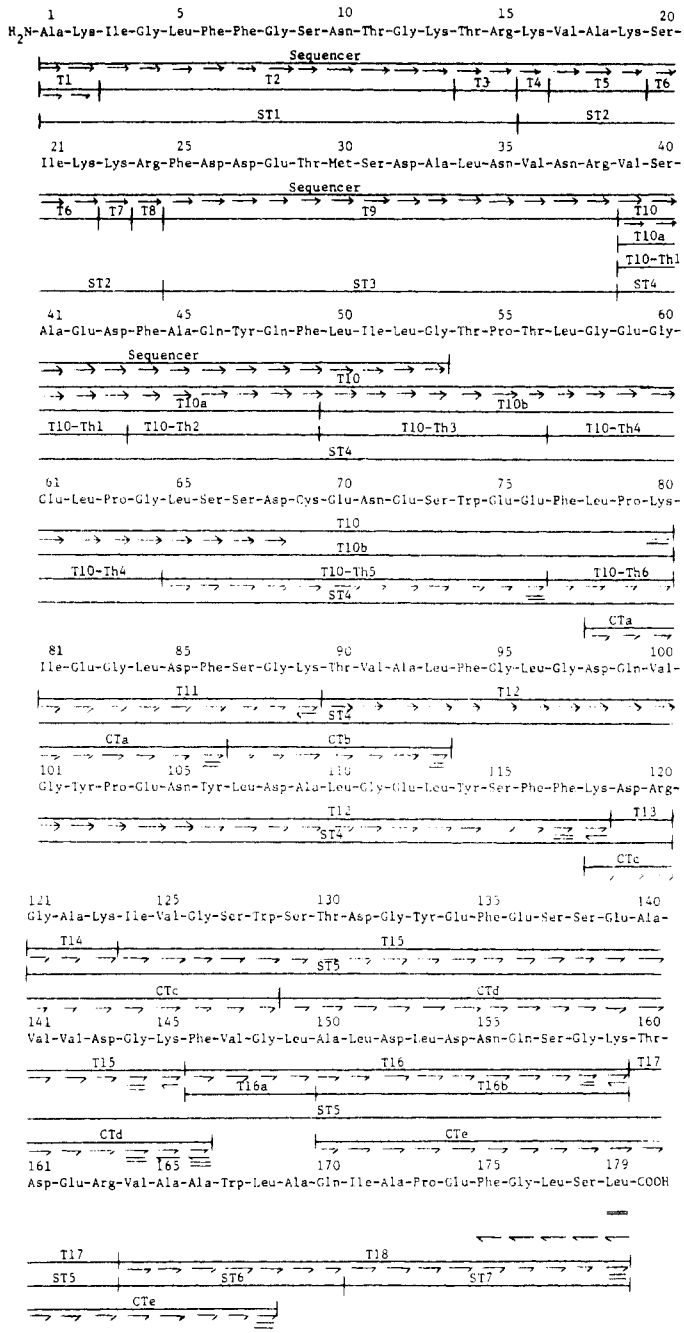


FIGURE 6: Reconstruction of the complete amino acid sequence of *Azotobacter* flavodoxin (azotoflavin). This figure summarizes (i) Protein Sequencer (Sequencer) results, (ii) two kinds of tryptic peptides (T and ST) obtained from the carboxymethyl derivative of the protein and succinylated carboxymethylazotoflavin, (iii) chymotryptic peptides (CT) necessary to establish the overlapping between tryptic peptides, and (iv) thermolytic peptides (T10-Th) obtained from peptide T10. In the figure, the symbols →, —, ═, and ⟞ represent sequence determined by Sequencer analysis, manual Edman degradation, hydrazinolysis, and carboxypeptidase A experiments, respectively, and, also, only the sequence results described in this text are indicated by the symbols.

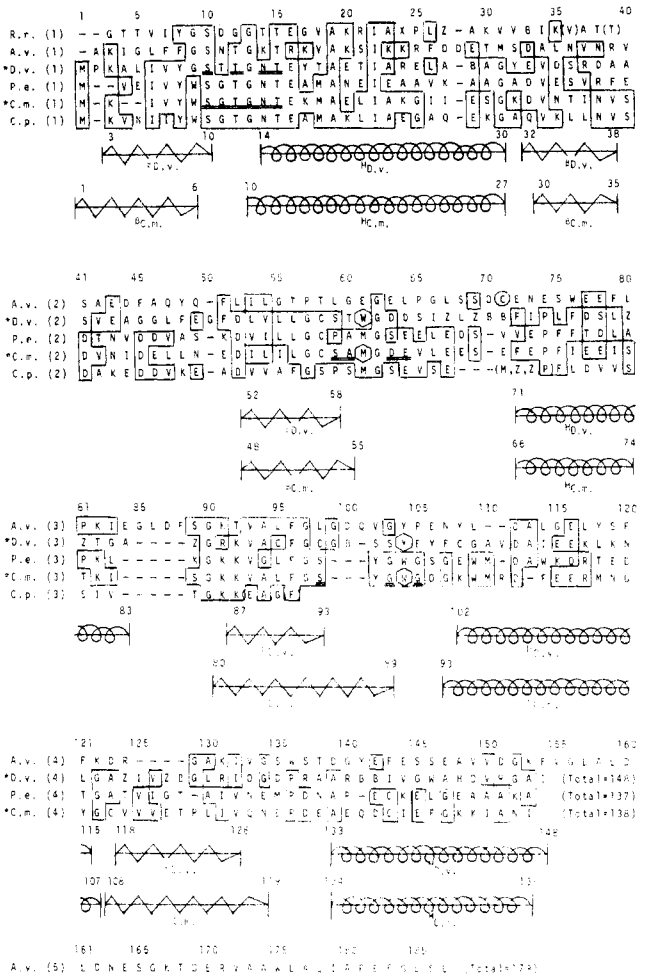


FIGURE 7: Comparison of the amino acid sequences of the group I (D.v., P.e., C.m., and C.p.) and group II (R.r. and A.v.) flavodoxins. Gaps have been introduced to obtain maximum sequence and conformational homology of the various flavodoxins. In the figure, the abbreviations R.r., A.v., D.v., P.e., C.m., and C.p. stand for the flavodoxins from *Rhodospirillum rubrum, A. vinelandii, Desulfovibrio vulgaris, Peptostreptococcus elsdenii, Clostridium MP.* and *Clostridium pasteurianum*, respectively. The symbols ⟞ and ⟞ stand for α-helical and β-sheet regions. In the figure, the one-letter code for amino acids has been adopted. An asterisk indicates that the crystal x-ray diffraction studies have been completed on the protein. The hexagon shows part of the FMN binding site. Double underlines show hydrogen-bonding sites with various portions of the FMN moiety. The circle shows the cysteine residue which is involved in the dimerization of two flavoprotein molecules.

this couple is +50 mV for the flavodoxin from strain O, whereas Yoch (1972) reported a potential of −270 mV for strain OP (Berkeley) flavodoxin. Because these differences in oxidation–reduction properties may be a reflection of a difference in sequence, it will be of considerable interest to compare the sequence of the strain O flavodoxin by MacKnight et al. (1974) with that of our strain.

The *A. vinelandii* flavodoxin was shown to consist of 179 amino acid residues and to be a single polypeptide chain protein with no disulfide bridges present. There is 1 cysteine residue present at position 69 (in Figure 6) which must be fairly close to the surface of the protein, since Yoch et al. (1975) have shown that there is a dimerization of two flavodoxin molecules through the conversion of 2 cysteine residues to a cystine residue, a process which results in the inactivation of biological activity.

The amino acid sequences of some flavodoxins are compared in Figure 7. The alignment was based on similar amino acid sequences as well as conformation, the latter of which comes from the crystal x-ray diffraction studies of Watenpaugh et al. (1973) and Burnett et al. (1974). Gaps were inserted wherever necessary to obtain maximum homology. The similarity in the NH_2-terminal sequences of the flavodoxins has already been discussed by MacKnight et al. (1974) and further discussions of this region will not be made. Similarities in amino acid sequences of the group I and II flavodoxins are indicated by squares in Figure 7 and these are distributed throughout the molecules. There are two residues flanking both

sides of the planar isoalloxazine ring in the *Clostridium* MP and *D. vulgaris* flavodoxins. These are a tryptophan or a methionine residue (position 61 according to the alignment in Figure 7) and a tyrosine or a tryptophan residue (position 104 in Figure 7). In the *A. vinelandii* flavodoxin, these are probably a glutamic acid residue (position 61) and a tyrosine residue (position 104). The region which contains amino acid 89–98 (Figure 7) shows great sequence homology and precedes the tyrosine or tryptophan (residue 104) which is within Van der Waals contact with the planar isoalloxazine ring. Despite the limitations in the Chou–Fasman procedure (Chou and Fasman, 1974) for the calculation of protein conformation from amino acid sequence data, considerable conformational similarities in the flavodoxins are predicted by this procedure (data not shown here). The sequence and conformation data are taken to indicate that the *A. vinelandii* flavodoxin and the group I flavodoxins have all arisen from a common ancestor and the main differences between the group I and II flavodoxins are differences in the COOH-terminal region in the group II flavodoxins which are longer by 31 or more amino acids. Crystal x-ray diffraction studies on another group II flavodoxin, the *Anacystis nidulans* flavodoxin, are underway in the laboratory of Dr. Martha Ludwig at the University of Michigan and, when these studies are completed, the conformational and the three-dimensional structure of a group II flavodoxin will soon be available.

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