the free SH groups in proteins. The most desirable of the reagents from this point of view is *p*-nitrophenylsulfenyl chloride.

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References

Anfinsen, C. B., and Haber, E. (1961), *J. Biol. Chem. 236*, 1361.
Bernfield, M. R. (1965), *J. Biol. Chem. 240*, 4753.
Ellman, G. L. (1958), *Arch. Biochem. Biophys. 74*, 443.
Ellman, G. L. (1959), *Arch. Biochem. Biophys. 82*, 70.
Gundlach, H. G., Stein, W. H., and Moore, S. (1959), *J. Biol. Chem. 234*, 1754.
Hirs, C. H. W., Moore, S., and Stein, W. H. (1953), *J. Biol. Chem. 200*, 493.
Hubacher, M. H. (1943), Organic Syntheses, Coll. Vol. 2, New York, N. Y., Wiley, p 445.
Kharasch, N., Gleason, G. I., and Buess, C. M. (1950), *J. Am. Chem. Soc. 72*, 1796.
Kunitz, M. (1946), *J. Biol. Chem. 164*, 563.
Parker, A. J., and Kharasch, N. (1960), *J. Am. Chem. Soc. 82*, 3071.
Porn, S. C., and Dutcher, J. T. (1956), *Anal. Chem. 38*, 836.
Sakakibara, S., and Tani, H. (1956), *Bull. Chem. Soc. Japan 29*, 85.
Scoffone, E., Fontana, A., and Rocchi, R. (1968), *Biochemistry 7*, 971 (this issue; preceding paper).
Smyth, D. G., Stein, W. H., and Moore, S. (1963), *J. Biol. Chem. 238*, 227.
Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.
Stracher, A. (1964), *J. Biol. Chem. 239*, 1118.
Tarbell, D. S., and Harnish, D. P. (1951), *Chem. Rev. 49*, 11.
Wellner, D., Silman, H. I., and Sela, M. (1963), *J. Biol. Chem. 238*, 1324.
Zincke, Th., and Lenhardt, S. (1913), *Ann. 400*, 2.

Nonheme Iron Proteins. IV. Structural Studies of *Micrococcus aerogenes* Rubredoxin*

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ABSTRACT: The *S*-β-aminoethylcysteinyl derivative of *Micrococcus aerogenes* rubredoxin was digested with trypsin and chymotrypsin in separate experiments. The fragments were purified and their sequences were determined by standard procedures. From the sequences of the individual peptides and the overlaps between them, the total amino acid sequence of the *M. aerogenes* rubredoxin was deduced. The stability of the chelate structure of this protein in dilute acids, 8 M urea, 50% ethanol, and 2.5 and 5 M guanidine hydrochloride was determined with respect to time. These studies and the resistance of the native protein to proteolytic digestion suggest a compact and stable confirmation for the native protein.

The nonheme iron protein, rubredoxin, was first crystallized from extracts of *Clostridium pasteurianum* by Lovenberg and Sobel (1965). Similar proteins have also been purified from several other anaerobic bacteria (Stadtman, 1965; Mayhew and Peel, 1966; Le Gall and Dragoni, 1966; Lovenberg, 1966; Bachmayer *et al.*, 1967a). In addition, Peterson *et al.* (1966) have isolated a rubredoxin-like protein from an aerobic bacterium, *Pseudomonas fluorescens*, and showed that this protein was necessary for ω hydroxylation of certain fatty acids.

All the rubredoxins isolated thus far have been reported to contain 1 g-atom of nonheme iron/mole of protein but no inorganic sulfide. The molecular weight of rubredoxin is approximately 6000 (Lovenberg and Sobel, 1965).

The present report describes the determination of the complete amino acid sequence of *Micrococcus aerogenes* rubredoxin. Observations on the stability of rubredoxin, the action of proteases on the native protein, and discussion concerning the possible iron ligands are included. Short communications of parts of the

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present work have been published previously (Bachmayer *et al.*, 1967a–c).

Experimental Section

Materials and Methods. Rubredoxin was prepared from *M. aerogenes* grown in a complex medium containing glutamate as described previously (Bachmayer *et al.*, 1967a). The samples used for the sequence studies showed a 280:490 absorbency ratio of about 2.4–2.5. Material used for the iron binding studies had an absorbency ratio of 2.4 or slightly lower.

Preparation of S-β-Aminoethylcysteinylrubredoxin. The iron was removed from native rubredoxin by trichloroacetic acid precipitation at 4° as reported for ferredoxin (Tanaka *et al.*, 1964). Specifically, to 10 ml of rubredoxin solution (2 mg/ml) was added 10 ml of 20% (w/v) trichloroacetic acid. The white precipitate was washed successively with 10% trichloroacetic acid (twice), 95% ethanol, and finally ether. The resulting aporubredoxin was dissolved in 8 M urea after adjusting the pH to 8.5 with *N*-ethylmorpholine. Aminoethylation was carried out by reaction with ethylenimine (Raftery and Cole, 1963). The resulting *S*-β-aminoethylcysteinylrubredoxin (AEC-rubredoxin)[1] was freed of reagents and urea by passing the reaction mixture through a column of Sephadex G-25 equilibrated with water. The eluted AEC-rubredoxin was lyophilized and stored.

Enzymatic Hydrolysis of AEC-rubredoxin. HYDROLYSIS BY TRYPSIN. About 7 μmoles of AEC-rubredoxin was suspended in 6 ml of water and dissolved by raising the pH to 8.0 with 0.1 N NaOH. Digestion was performed in a constant-temperature chamber compartment (37°). At zero time, 2% (w/w) of L-(tosylamido-2-phenyl)ethyl chloromethyl ketone treated trypsin (TPCK-trypsin) (Wang and Carpenter, 1965) was added and another 2% after 3 hr. After 8-hr reaction, the reaction mixture was lyophilized. An aliquot of the digestion mixture was subjected to high-voltage paper electrophoresis at pH 6.5 (pyridine–acetic acid) in the first dimension and paper chromatography in 1-butanol–pyridine acetic acid–water (15:10:3:12, v/v) in the second dimension.

CHYMOTRYPTIC HYDROLYSIS. Another 7 μmoles of AEC-rubredoxin was digested with L-(tosylamido-2-lysyl)ethyl chloromethyl ketone treated chymotrypsin (TLCK-chymotrypsin) (Mares-Guia and Shaw, 1963). At zero time, 2% (w/w) enzyme was added and after 3 hr another 2% was added. The hydrolysis was allowed to proceed for 16 hr at 37°.

Column Chromatographic Separation of Tryptic and Chymotryptic Peptides. Chromatography was performed on 1.5 × 120 cm columns of Dowex AG-1-X2 (Bio-Rad Laboratory) which had been equilibrated with pyridine–collidine–acetic acid buffer of pH 7.8 (Schroeder *et al.*, 1962). The lyophilized reaction mixtures of the tryptic and chymotryptic digest were dissolved in 6–10 ml of starting buffer. Small amounts of insoluble material were removed by centrifugation and washed twice with the starting buffer. The combined washings and solution were applied to the column and gradient elution was performed according to Schroeder *et al.* (1962).

For the separation of the tryptic peptides, the elution was initiated with a gradient of 500 ml of buffer (pyridine–collidine–acetic acid, pH 7.8) in the mixing chamber and 500 ml of 0.4 M acetic acid in the reservoir. Elution was continued with a gradient consisting of 100 ml of 0.1 M acetic acid in mixing chamber and 100 ml of 0.4 M acetic acid in the reservoir. Then, stepwise elution with 0.4 M acetic acid (200 ml), 1 M acetic acid (200 ml), and 50% acetic acid (v/v) (200 ml) was performed.

Fractions of 100 drops each (about 3 ml) were collected. Aliquots of 50 μl were removed from every fraction and treated with ninhydrin (Moore and Stein, 1948). In the case of the chymotryptic digest, elution was started with 60 ml of buffer (pyridine–collidine–acetic acid, pH 7.8). A gradient consisting of 500 ml of buffer in the mixing chamber and 500 ml of 0.2 M acetic acid in the reservoir was used. After the reservoir was empty, another 500 ml of 0.2 M acetic acid was added. Then, stepwise elution with 330 ml of 0.1 M acetic acid, 250 ml of 0.2 M acetic acid, 250 ml of 0.4 M acetic acid, 400 ml of 1 M acetic acid, and finally 300 ml of 50% (v/v) acetic acid was performed.

Purity Checks and Further Purification of Peptide Fractions. Each fraction was checked for purity by high-voltage paper electrophoresis using pyridine–acetate buffer (pH 6.5) and by paper chromatography using *n*-butyl alcohol–pyridine–acetic acid–water (15:10:3:12, v/v) or *n*-butyl alcohol–acetic acid–water (4:1:5, v/v) as solvent systems. The chromatograms were developed with a 0.2% ninhydrin spray in acetone. After noting the ninhydrin-positive spots, the papers were sprayed with Pauly's reagent (Smith, 1960) to locate the tyrosine-containing peptides.

Tryptophan peptides were detected on separate chromatograms by using Ehrlich's reagent (Smith, 1960). Depending on the results of the purity checks of the individual fractions, further purification when necessary was accomplished by one or a combination of the following methods: chromatography on Dowex 50-X2, gel filtration on Sephadex G-25 columns using 20% acetic acid as solvent, and preparative paper chromatography on Whatman No. 3MM paper with the solvent systems described above. For purification on Dowex 50-X2, the resin was equilibrated with 0.2 M pyridine–acetic acid buffer of pH 3.1. Unless otherwise stated, 1 × 50 cm columns were used and the elution was performed with a gradient consisting of 250 ml of 0.2 M pyridine–acetic buffer (pH 3.1) in the mixing chamber and 250 ml of 2 M pyridine–acetic buffer (pH 5.0) in the reservoir (Schroeder *et al.*, 1962).

Hydrolysis of Peptides with the Bacillus subtilis Neutral Proteases. One of the peptides, peptide C-8a, was further digested with the *B. subtilis* neutral protease (McConn *et al.*, 1964). In this procedure, 1 μmole of

[1] Abbreviations used: AEC, *S*-β-aminoethylcysteinyl; PTH, phenylthiohydantoin; TPCK, L-(tosylamido-2-phenyl)ethyl chloromethyl ketone; LAP, leucine aminopeptidase; Cpase, carboxypeptidase.

FIGURE 1: Crystals of *M. aerogenes* rubredoxin obtained 70–80% saturation with ammonium sulfate. 25× magnification.

the peptide was incubated with 2% (w/w) of the *B. subtilis* neutral protease in 2 ml of 0.01 M ammonium acetate buffer (pH 7.0). The reaction was allowed to proceed for 18 hr at 38°, and then terminated by boiling. The sample was dried *in vacuo*. The peptides were separated by paper chromatography on Whatman No. 3MM paper using the solvent system *n*-butyl alcohol–pyridine–acetic acid–water (15:10:3:12, v/v). The individual peptides were localized by spraying with dilute ninhydrin (0.05%) in acetone and the residual peptide was eluted from the paper with 20% acetic acid.

Amino Acid Analysis. Peptides were hydrolyzed with 6 N HCl (glass distilled) at 105° for 20–24 hr routinely but up to 72 hr for acid-resistant peptides. Phenol was added to the sample and the tubes were sealed *in vacuo*. After hydrolysis, HCl was removed with a stream of nitrogen at 40°. Amino acid analyses were performed on a Spinco Model 120 automatic amino acid analyzer (Spackman *et al.*, 1958). The results of the amino acid analyses are given as molar ratios. In the case of the methionine-containing peptides, performic acid oxidation (Moore, 1963) was performed prior to acid hydrolysis.

Determination of Tryptophan Content. The tryptophan content of the aporubredoxin was determined by the spectrophotometric method of Goodwin and Morton (1946) and the reaction with *N*-bromosuccinimide (Witkop, 1961).

Dinitrophenylation. The procedure as described by Fraenkel-Conrat *et al.* (1955) was used. Dinitrophenyl-amino acids (DNP-amino acids) were identified by thin-layer chromatography (Randerath, 1966).

Hydrazinolysis. The procedure of Bradbury (1958) was used to liberate the COOH-terminal amino acid which was identified and quantitated on the amino acid analyzer.

Leucine Aminopeptidase (LAP). LAP (Worthington Biochemical Corp.) was used after pretreatment with diisopropylfluorophosphate. Before use, the enzyme was activated by incubation with 0.005 M $MgCl_2$ at pH 8.0 (Tris-HCl) for 30–60 min at 40°. In the reaction with peptides, an enzyme concentration of 5–10% (w/w) was used and the reactions were carried out at pH 8.0 at 40° for 16–24 hr.

Carboxypeptidase B (CPase B). Diisopropylfluorophosphate-treated enzyme (Worthington Biochemical Corp.) was used. Digestions were carried out at pH 8.0 (0.02 M *N*-ethylmorpholine–HCl) and 40° at an enzyme:peptide ratio of 1:20 to 1:10 (w/w) for varying lengths of time.

Carboxypeptidase A (CPase A). The diisopropylfluorophosphate-treated enzyme (Worthington) was stored as a suspension in water in the refrigerator. Prior to use, the crystals were washed twice with water in the cold. The crystalline suspension was then solubilized with 0.1 N NaOH and the pH was immediately lowered to 8.0 with 0.1 N acetic acid. The conditions for the digestions were the same as described for carboxypeptidase B.

Edman Degradation. The subtractive procedure as described by Konigsberg and Hill (1962) was used, but all reactions were carried out under nitrogen. For the direct identification of the PTH-amino acids, thin-layer chromatography was used (Randerath, 1966).

Cleavage with Cyanogen Bromide (Witkop, 1961). The sample, dissolved in 0.2 N acetic acid or 70% formic acid, was treated with a 50-fold M excess of CNBr for 18 hr at 40°.

Nomenclature. The "C" or "T" placed before the peptide number indicates that the peptide was obtained from a chymotryptic or tryptic digest, respectively. Peptides are numbered in the order of their elution from the Dowex AG 1-X2 column. The peptides with a "P" within the symbol were obtained by further digestion of peptide C-8a with the *B. subtilis* neutral protease.

Results

General Studies

Purification of M. aerogenes Rubredoxin. Rubredoxin from *M. aerogenes* was purified to a state of crystallinity. Figure 1 shows a photograph of crystals obtained by the addition of ammonium sulfate to preparations with a 280:490 absorbance ratio of 2.4.

Spectral Properties. The absorption spectrum of rubredoxin from *M. aerogenes* showed no great differences from spectra obtained for rubredoxins isolated from other species (Stadtman, 1965; Mayhew and Peel, 1966; Le Gall and Dragoni, 1966; Lovenberg, 1966). The molar absorptivity indices were found to be: 18.3, 8.4, 9.2, 7.65, and 3.5×10^3 for the maxima at 280, 350, 378, 490, and 570 mμ, respectively (protein concentrations were determined by amino acid analyses of an acid hydrolysate).

TABLE I: Amino Acid Composition and Content of Iron and "Labile Sulfur" of Rubredoxin and Ferredoxin.

Amino Acid	Rubredoxin from *M. aerogenes*[a]	Rubredoxin from *C. pasteurianum*[b]	Ferredoxiin from *C. pasteurianum*[c]
Lysine	2.0(2)	4	1
Histidine	nd(0)	0	0
Arginine	nd(0)	0	0
Cysteine	3.6(4)	4	8
Aspartic acid	8.0(8)	12	8
Threonine	1.9(2)	3	1
Serine	1.1(1)	0	5
Glutamic acid	8.1(8)	6	4
Proline	3.7(4)	5	3
Glycine	4.9(5)	6	4
Alanine	2.9(3)	0	8
Valine	3.9(4)	5	6
Methionine	0.8(1)	1	0
Isoleucine	0.9(1)	2	5
Leucine	2.8(3)	1	0
Tyrosine	2.7(3)	3	1
Phenylalanine	2.9(3)	2	1
Tryptophan[d]	(1)	2	0
Total number of amino acid residues	53	56	55
Fe	1	1	7
Labile sulfur	0	0	7

[a] Average values from several analyses of AEC derivatives hydrolyzed for 12, 24, 48, and 72 hr. The values for Ser and Thr are those obtained by extrapolating to zero time. In independent experiments using a performic acid oxidized sample, 3.7 residues of cysteic acid were determined; numbers in parentheses represent residues established by sequence studies; nd = not detectable. [b] Lovenberg (1966). [c] Tanaka *et al.* (1964). [d] Tryptophan content was determined by the spectrophotometric method of Goodwin and Morton (1946) and by titration with *N*-bromosuccinimide, using the iron-free protein.

The ultraviolet absorption of native rubredoxin is much higher than one would expect by adding up the increments for three Tyr's and one Trp per mole. The expected theoretical molar extinction coefficient at 280 mμ would be 9.6×10^3, compared to the experimental value of 18.3×10^3 found for rubredoxin. A similar phenomenon was observed for ferredoxins (Keresztes-Nagy and Margoliash, 1966) and was explained by a contribution of the chelate structure to the ultraviolet absorption. With AEC-rubredoxin, which does not contain iron and probably has an altered tertiary structure, the comparable extinction coefficient was found to be 8.5×10^3 at 280 mμ.

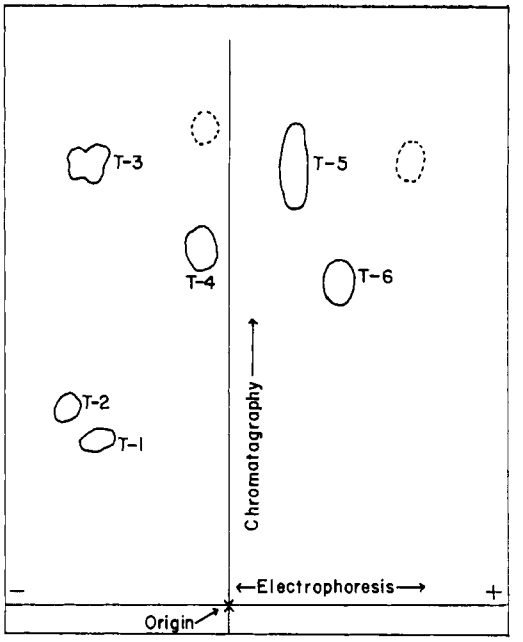

FIGURE 2: Fingerprint map of the tryptic digest of AEC-rubredoxin. High-voltage paper electrophoresis at pH 6.5 was used in the first dimension, followed by paper chromatography in 1-butanol–pyridine–acetic acid–water (15:10:3:12, v/v) in the second dimension. The spots are those obtained after spraying with ninhydrin.

Amino Acid Composition of Rubredoxins. Table I shows the amino acid composition of *M. aerogenes* rubredoxin together with the values for rubredoxin and ferredoxin from another anaerobic bacterium, *C. pasteurianum*. All of the proteins in Table I lack the amino acids histidine and arginine and have a remarkably high content of acidic amino acids. Furthermore, rubredoxins from *Peptostreptococcus elsdenii* (Mayhew and Peel, 1966), *M. lactilyticus*, and *C. pasteurianum* (Lovenberg, 1966) have been reported to contain four cysteine residues and three tyrosine residues.

End Groups of the Intact Protein. After dinitrophenylation of the performic acid oxidized aporubredoxin, DNP-methionine sulfone was identified in the water phase of acid hydrolysates by thin-layer chromatography. DNP-glycine and DNP-proline were not detected in 12 N HCl hydrolysates of the DNP protein. Digestion with leucine aminopeptidase yielded methionine and glutamine in 20 and 16% yield, respectively, even when the iron-containing native protein was used. Neither carboxypeptidase A nor B (or a mixture of both) liberated reasonable amounts of any amino acid. However, hydrazinolysis yielded aspartic acid in 50% yield fixing it in the carboxyl-terminal position of the protein.

Separation and Purification of the Tryptic Peptides

Fingerprint Map and Purification of the Tryptic Peptides. The fingerprint map of an aliquot of the tryptic digest is shown in Figure 2. Column chromatography of the tryptic peptides was performed on Dowex 1-X2 and the elution pattern is shown in Figure 3. Peptides T-1, T-2, T-3, and T-4 proved to be sufficiently pure to be used for sequence studies without further purifi-

TABLE II: Amino Acid Composition of Tryptic Peptides.

Amino Acid	T-1	T-2	T-3	T-4	T-5[b]	T-6	Total	Original Protein
Lysine	1.10(1)	1.00(1)					2	2
AEC			0.85(1)	0.92(1)	1.40(2)		4	4
Aspartic acid					6.15(6)	2.00(2)	8	8
Threonine			1.00(1)		0.75(1)		2	2
Serine					0.77(1)		1	1
Glutamic acid		1.07(1)		1.00(1)	3.10(3)	2.90(3)	8	8
Proline					3.65(4)		4	4
Glycine	1.85(2)				2.90(3)		5	5
Alanine	1.05(1)				1.98(2)		3	3
Valine					2.78(3)	0.80(1)	4	4
Methionine		0.94(1)					1	1
Isoleucine					0.91(1)		1	1
Leucine			0.92(1)		1.84(2)		3	3
Tyrosine					1.72(2)	0.73(1)	3	3
Phenylalanine				0.88(1)	1.00(1)	0.88(1)	3	3
Tryptophan[a]					(1)		1	1
Total	4	3	3	3	32	8	53	53

[a] The presence of tryptophan was shown by a positive Ehrlich reaction. In addition, a tyrosine:tryptophan ratio of 1.9:1 (two determinations) was found for peptide T-5 by the spectrophotometric method of Goodwin and Morton (1946). [b] Hydrolysis time was 70 hr.

cation. Peptide T-5 was purified on Dowex 50-X2 and peptide T-6 by gel filtration on Sephadex G-25 in 20% acetic acid.

Amino Acid Composition and Properties of the Purified Tryptic Peptides. The amino acid composition of the purified peptides, their electrophoretic properties, R_F values during paper chromatography, and special staining reactions are summarized in Tables II and III.

Amino Acid Sequence of the Tryptic Peptides

Peptide T-2-Met-Gln-Lys (Residues 1–3). Carboxypeptidase B liberated Lys in 87% yield. Leucine aminopeptidase digestion yielded Met and Gln in 20 and 17% yield, respectively (Lys was not determined). DNP-methionine sulfone was identified in an acid hydrolysate of a performic acid oxidized sample of the peptide.

The peptide was also cleaved with CNBr and the fragments were separated by thin-layer chromatography. Fragment T-2-CNBr 1 had the composition Lys, 1.00(1); Glu, 1.29(1); in fragment T-2 CNBr 2 only homoserine could be detected in reasonable amounts. All these results together with the fact that the peptide was basic (paper electrophoresis at pH 6.5) confirmed the sequence shown above.

Peptide T-4-Phe-Glu-AEC (Residues 4–6). DNP-Phe was identified after dinitrophenylation. AEC (66%) was found to be released with carboxypeptidase B. After steps 1 and 2 of Edman degradation PTH-Phe and PTH-Glu were identified by thin-layer chromatography. The fact that this peptide is neutral confirms that it contains Glu and not Gln.

Peptide T-3-Thr-Leu-AEC (Residues 7–9). Dinitrophenylation gave Thr as NH_2-terminal residue. Carboxypeptidase B digestion liberated 88% AEC. Two steps of Edman degradation gave the following results: step 1: AEC, 0.83(1); *Thr, 0.15(0)*; Leu, 0.95(1); step 2: AEC, 0.80(1); Thr, 0.12(0); *Leu, 0.20(0)*.

Peptide T-5-Gly-Tyr-Ile-(AEC, Asp_6, Thr, Ser, Glu_3, Pro_4, Gly_2, Ala_2, Val_3, Leu_2, Tyr, Phe, Trp)-AEC (Residues 10–41). The values for AEC were unusually low in 24-hr hydrolysates of this peptide and the results are possibly due to the occurrence of Val-AEC, Leu-AEC, and Tyr-Ile-Tyr linkages. However, an increase of the hydrolysis time to 70 hr increased the tyrosine content but had no effect on the AEC content. This may be due to a partial destruction of aminoethylcysteine after preparation of the derivative since the AEC content of the AEC-protein was 3.6 residues. Moreover, since T-5 contains parts of C-1 and C-4, each of which contain AEC, it is obvious that T-5 should contain two AEC residues. Only the terminal parts of the sequence have been determined for this peptide in order to make the placements of the chymotryptic peptides possible. Dinitrophenylation gave DNP-Gly. With carboxypeptidase B only 13% AEC could be released, even when 15% enzyme (w/w) was used. This is probably due to the fact that Pro is in the third position from the COOH terminus, as determined by sequence analysis of the chymotryptic peptides. During steps 1–3 of the Edman degradation

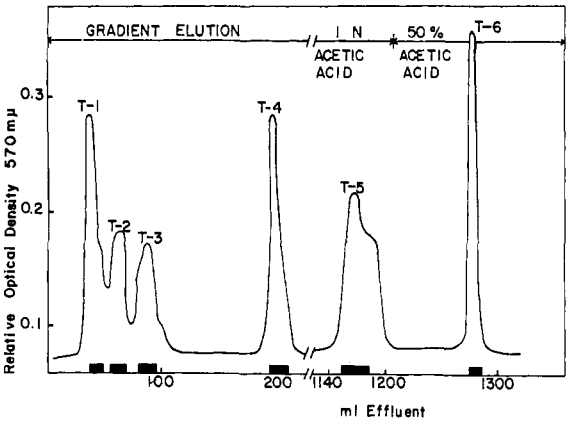

FIGURE 3: Dowex AG-1-X2 elution pattern of the tryptic digest of AEC-rubredoxin. The digestion mixture of 7 μmoles of protein was applied to a Dowex AG-1-X2 column (1.5 × 120 cm) and gradient elution was carried out as described in the Experimental Section.

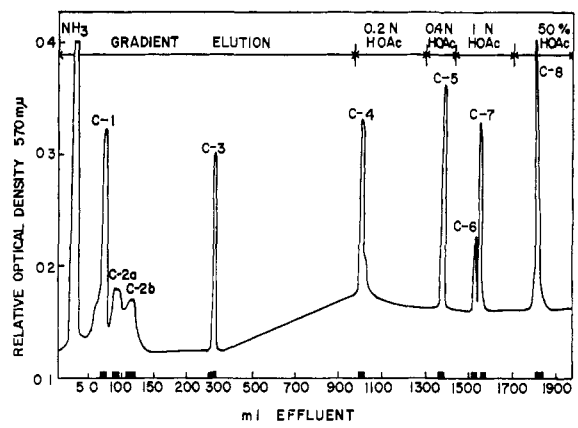

FIGURE 4: Dowex AG-1-X2 elution pattern of the chymotryptic digest of AEC-rubredoxin (7 μmoles). A 1.5 × 120 cm column was used and gradient elution was performed as described in the Experimental Section.

reaction, PTH-Gly, PTH-Tyr, and PTH-Ile were directly identified.

Peptide T-1-Gly-Ala-Gly-Lys (Residues 42–45). After dinitrophenylation DNP-Gly was identified as the NH_2-terminal residue. Carboxypeptidase B released lysine in 99% yield. Two steps of Edman degradation gave the following results: step 1: Lys, 1.00(1); *Gly, 1.31(1)*; Ala, 1.05(1); step 2: Lys, 0.95(1); Gly, 1.28(1); *Ala, 0.33(0)*.

Peptide T-6-Glu-Asp-Phe-Glu-Val-Tyr-Glu-Asp (Residues 46–53). Hydrazinolysis demonstrated that aspartic acid (40% yield) was the COOH-terminal amino acid. Six steps of Edman degradation yielded the following results: step 1: Asp, 2.00(2); *Glu, 2.18(2)*; Val, 0.82(1); Tyr, 0.80(1); Phe, 0.91(1); step 2: *Asp, 1.23(1)*; Glu, 1.99(2); Val, 0.85(1); Tyr, 0.67(1); Phe, 0.74(1); step 3: Asp, 1.07(1); Glu, 2.00(2); Val, 0.83(1.0); Tyr, 0.70(1); *Phe, 0.21(0)*; step 4: Asp, 1.00(1); *Glu, 1.21(1)*; Val, 0.71(1); Tyr, 0.64(1); Phe, 0.15(0); step 5: Asp, 1.00(1); Glu, 1.20(1); *Val, 0.22 (0)*; Tyr, 0.65(1); Phe, 0.18(0); step 6: Asp, 1.00(1); Glu, 1.23(1); Val, 0.25(0); *Tyr, 0.26(0)*; Phe, 0.17(0). During steps 1–4, PTH-Glu, PTH-Asp, PTH-Phe, and PTH-Glu, respectively, were identified. Amide analysis of the acid hydrolysate of the peptide indicated no amide was present. Blanks for NH_3 were run on an equivalent amount of acid used for hydrolysis, and the sample dilution buffer, as well as the sample itself.

TABLE III: Properties of Tryptic and Chymotryptic Peptides.

Peptide	R_F[a]	Electrophoretic Properties[b]	Special Staining Reactions
T-1	0.13	Basic	
T-2	0.18	Basic	
T-3	0.39	Basic	
T-4	0.29	Neutral	
T-5	0.42–44	Acidic	Pauly+; Ehrlich+
T-6	0.28	Acidic	Pauly+
C-1	0.67	Basic	
C-2	0.52	Basic	
C-3a	0.49	Neutral	
C-3b	0.38	Basic	Pauly+
C-4	0.36	Weak acidic	Pauly+
C-5	0.26	Weak acidic	Pauly+
C-6	0.57	Acidic	Ehrlich+
C-7	0.19	Acidic	
C-8a	0.54–55	Acidic	Pauly+
C-8b	0.33–34	Acidic	Ehrlich+

[a] Chromatography on Whatman No. 1 filter paper, solvent system *n*-butyl alcohol–pyridine–acetic acid–water (15:10:3:12, v/v). [b] High-voltage paper electrophoresis on Whatman No. 3MM paper, in pyridine–acetic acid–water (100:4:900, v/v), pH 6.5.

Separation and Purification of the Chymotryptic Peptides

Figure 4 shows the elution pattern of the chymotryptic peptides. The contents of the major fractions were pooled as indicated by the solid bars. Purification of all the chymotryptic peptides were accomplished by chromatography on Dowex 50-X2 columns. The elution patterns for peptides C-3 and C-8 from the Dowex 50 columns are shown in Figures 5 and 6. All other peptides gave only one major ninhydrin-positive peak.

Tables III and IV demonstrate the amino acid composition of the chymotryptic peptides obtained after the purification steps together with their electrophoretic and chromatographic properties.

Amino Acid Sequence of the Various Chymotryptic Peptides

Peptide C-2-Met-Gln-Lys-Phe (Residues 1–4). A double peak was obtained on elution from the Dowex 1-X2 column. Since the amino acid analysis of the two peptides (C-2a and C-2b) (Figure 3) gave the same composition, the two peaks were pooled and purified together on a column of Dowex 50-X2.

Carboxypeptidase A released phenylalanine in 75%

TABLE IV: Amino Acid Composition of Chymotryptic Peptides.

Amino Acid	C-1	C-2	C-3a	C-3b	C-4	C-5	C-6	C-7	C-8a	C-8b	Total[a]	Original Protein
Lysine		1.00(1)			1.00(1)	1.10(1)					2	2
AEC	0.90(1)		0.90(1)	1.00(1)	0.98(1)	1.10(1)	0.91(1)				4	4
Aspartic acid					1.00(1)	1.90(2)	2.01(2)	1.00(1)	4.07(4)	1.86(2)	8	8
Threonine			0.95(1)						0.88(1)		2	2
Serine							1.17(1)			1.11(1)	1	1
Glutamic acid		1.13(1)	1.05(1)		2.03(2)	2.98(3)	1.78(2)	1.01(1)	0.97(1)	2.08(2)	8	8
Proline	1.00(1)						1.05(1)		3.06(3)		4	4
Glycine				0.97(1)	2.04(2)	2.19(2)			2.28(2)		5	5
Alanine					0.98(1)	1.04(1)			2.02(2)		3	3
Valine	0.84(1)				0.89(1)	0.91(1)	1.94(2)		1.10(1)	0.94(1)	4	4
Methionine		0.88(1)									1	1
Isoleucine									0.75(1)		1	1
Leucine	1.00(1)		0.90(1)				1.00(1)		1.06(1)		3	3
Tyrosine				0.93(1)	0.78(1)	0.83(1)			0.80(1)		3	3
Phenylalanine		0.95(1)			0.80(1)	0.96(1)			0.93(1)		3	3
Tryptophan[b]							(1)			(1)	1	1
Total	4	4	4	3	11	13	11	2	18	7	53	53

[a] The numbers of amino acid residues in peptides C-1, C-4, C-7, and C-8b are not included since C-5 contains C-4 plus C-7, and C-6 is the sum of C-1 and C-8b. [b] The presence of tryptophan was shown by a positive Ehrlich reaction.

TABLE V: *B. subtilis* Neutral Protease Fragments of Peptide C-8a.

Peptide	Amino Acid Composition and Sequence Results
P-1	Ile-(Tyr,Asp,Pro)-Ala. Ile 0.84(1), Ala, 0.99(1), Asp 1.04(1), Pro, 1.00(1), Tyr 0.79(1). Dinitrophenylation yielded only DNP-Ile. One step of the Edman degradation yielded PTH-Ile. Hydrazinolysis liberated alanine.
P-2	Val-Gly-Pro-Asp-Thr-Pro-Asp-Gln-Asp-Gly. Asp 3.20(3), Thr 0.78(1), Glu 1.08(1), Pro 1.89(2), Gly 1.80(2), Val 0.96(1). The first eight sequences were determined by direct identification of the PTH-amino acid formed at each of the steps. Hydrazinolysis liberated glycine. Cpase A liberated after 16 hr 32% glycine and 25% aspartic acid.
P-3a	Ala-Phe. Ala 1.00, Phe 1.00. After one step of Edman reaction, PTH-ala was directly identified.
P-3b	Leu The sequence is: Ile-Tyr-Asp-Pro-Ala-Leu-Val-Gly-Pro-Asp-Thr-Pro-Asp-Gln-Asp-Gly-Ala-Phe

yield. Since there was only one methionine residue in the protein and the sequence of the methionine-containing peptide T-2 had been determined, the sequence of peptide C-2 was deduced.

Peptide C-3a-Glu-AEC-Thr-Leu (Residues 5–8). Digestion with carboxypeptidase A liberated 77% Leu and 11.9% Thr. Three steps of Edman degradation gave the following results: step 1: AEC, 0.88(1); Thr, 1.00(1); *Glu, 0.15(0)*; Leu, 1.02(1); PTH-Glu identified; step 2: *AEC, 0.06(0)*; Thr, 0.96(1); Glu, 0.12(0); Leu, 1.01(1); step 3: AEC, (not determined); *Thr, 0.10(0)*; Glu, 0.16(0); Leu, 1.00(1).

Peptide C-3b-AEC-Gly-Tyr (Residues 9–11). Carboxypeptidase A released Tyr in 60% yield. Two steps of Edman degradation confirmed the sequence above: step 1: *AEC, 0.07(0)*; Gly, 1.00(1); Tyr, 0.68(1); step 2: AEC, 0.10(0); *Gly, 0.47(0)*; Tyr, 0.70(1).

Peptide C-8a-Ile-Tyr-Asp-Pro-Ala-Leu-Val-Gly-Pro-Asp-Thr-Pro-Asp-Gln-Asp-Gly-Ala-Phe (Residues 12–29). The only NH_2-terminal amino acid found after dinitrophenylation was DNP-Ile. The sequence of this peptide was determined by Edman degradation, Cpase A digestions, and by sequence analyses of smaller fragments obtained by cleavage of the peptide with the *B. subtilis* neutral protease.

The results of six steps of the Edman degradation are summarized as follows: step 1: Asp, 4.15(4); Thr, 1.00(1); Glu, 1.28(1); Pro, 2.78(3); Gly, 2.15(2); Ala, 1.90(2); Val, 0.95(1); *Ile, 0.09(0)*; Leu; 0.95(1); Tyr, 1.02(1); Phe, 1.08(1); step 2: Asp, 4.05(4); Thr, 0.85(1); Glu, 1.30(1); Pro, 2.80(3); Gly, 1.99(2); Ala, 1.85(2); Val, 0.91(1); Ile, 0.08(0); Leu, 0.85(1); *Tyr, 0.07(0)*; Phe, 0.90(1); step 3: *Asp, 3.22(3)*; Thr, 0.83(1), Glu, 1.32(1); Pro, 2.78(3); Gly, 2.03(2); Ala, 1.75(2); Val, 1.01(1); Ile, 0.06(0); Leu, 1.02(1); Tyr, 0.05(0); Phe, 0.81(1); step 4: Asp, 3.18(3); Thr, 0.80(1); Glu, 1.00(1); *Pro, 1.87(2)*; Gly, 1.80(2); Ala, 1.70(2); Val, 0.78(1); Ile, 0.05(0); Leu, 0.76(1); Tyr, 0.05(0); Phe, 0.80(1); step 5: Asp, 3.08(3); Thr, 0.80(1); Glu, 1.34(1); Pro, 2.10(2); Gly, 1.95(2); *Ala, 1.14(1)*; Val, 0.93(1); Ile, 0.07(0); Leu, 0.94(1); Tyr, 0.04(0); Phe, 0.70(1). PTH-Ile, PTH-Tyr, PTH-Asp, PTH-Ala, and PTH-Leu were directly identified in steps 1, 2, 3, 5, and 6, respectively. Leucine aminopeptidase released Ile and Tyr in almost equal yields (56 and 55%, respectively). Digestion with Cpase A liberated after 1 hr, phenylalanine, alanine, and glycine in 90, 65, and 9%, respectively. A 16-hr digest

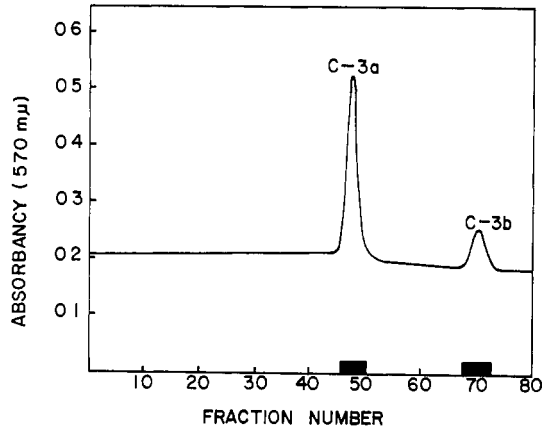

FIGURE 5: Dowex 50-X2 elution pattern of the chymotryptic fraction C-3. The column used was 1 × 50 cm and elution was performed with a gradient consisting of 250 ml of 0.2 M pyridine–acetate buffer (pH 3.1) and 2 M pyridine–acetate buffer (pH 5.0).

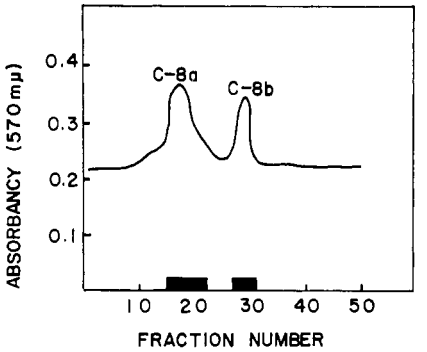

FIGURE 6: Dowex 50-X2 elution pattern of the chymotryptic fraction C-8. Gradient elution on a column 1 × 50 cm was carried out from 0.2 M pyridine–acetate buffer (pH 3.1) to 2 M pyridine–acetate buffer (pH 5.0).

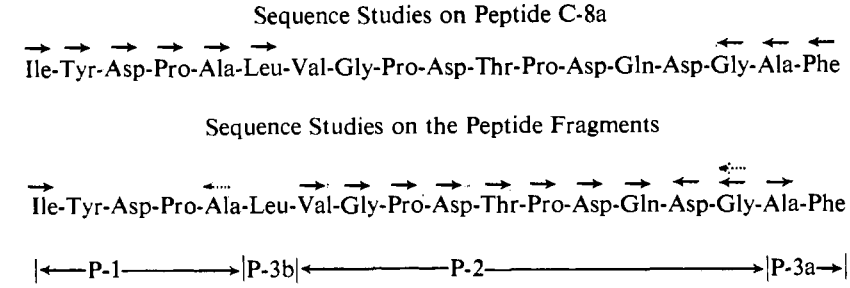

FIGURE 7: Construction of the amino acid sequence of peptide C-8a. The alignment of the protease fragments P-1 to P-3b is shown together with the sequence results obtained on the intact peptide. Arrows pointing to the right indicate Edman degradative reactions; arrows pointing to the left indicate Cpase A data; and dashed arrows pointing to the left indicate hydrazinolysis data.

yielded 93% phenylalanine, 71% alanine, 18.6% glycine, 11.1% aspartic acid, and about 10% of an amino acid which appeared in the position of serine during amino acid analysis (Gln or Asn). The fragments obtained by *B. subtilis* protease are summarized in Table V and Figure 7.

Peptide C-6-Glu-(AEC, Asp$_2$, Ser, Glu, Pro, Val$_2$, Trp)-Leu (Residues 30–40). The presence of tryptophan in this peptide was assumed from the positive reaction with Ehrlich reagent. Leu was determined as COOH-terminal amino acid by hydrazinolysis. One step of subtractive Edman degradation showed that Glu is the NH_2-terminal amino acid. The composition of the residual peptide was AEC, 0.68(1); Asp, 2.00(2); Ser, 0.99 (1); *Glu, 1.22(1)*; Pro, 1.19(1); Val, 1.75(2); Leu, 0.95(1). PTH-Glu was directly identified by thin-layer chromatography.

Peptide C-8b-Glu-Asp-Val-Ser-Glu-Asn-Trp (Residues 30–36). Only tryptophan was released by Cpase A. Five steps of the subtractive Edman degradation gave the following results: step 1: Asp, 2.00(2); Ser, 1.07(1); *Glu, 1.25(1)*; Val, 1.04(1); step 2: *Asp, 1.05(1)*; Ser, 0.96(1); Glu, 1.12(1); Val, 0.79(1); step 3: Asp, 0.96(1); Ser, 1.20(1); Glu, 1.05(1); *Val, 0.29(0)*; step 4: Asp, 0.99(1); *Ser, 0.47(0)*; Glu, 1.13(1); Val, 0.22(0); step 5: Asp, 1.00(1); Ser, 0.35(0); *Glu, 0.52(0)*; Val, 0.21(0). Tryptophan was not determined since the Cpase A experiment demonstrated that tryptophan was COOH terminal and from the known specificity of chymotrypsin tryptophan would also be COOH terminal. During steps 1, 2, 3, 5, and 6, PTH-Glu, PTH-Asp, PTH-Val, PTH-Glu, and PTH-Asn were directly identified.

Peptide C-1-Val-AEC-Pro-Leu (Residues 37–40). Leucine was the only free amino acid obtained after hydrazinolysis (35% yield). Three steps of Edman degradation gave the following results: step 1: AEC, 0.66(1); Pro, 1.00(1); *Val, 0.08(0)*; Leu, 0.91(1); step 2: *AEC, 0.15 (0)*; Pro, 1.00(1); Val, 0.10(0); Leu, 0.92(1); step 3: AEC, 0.16(0); *Pro, 0.25(0)*; Val, 0.09(0); Leu, 0.92(1).

Peptide C-5-AEC-(Lys, Asp, Glu$_3$, Gly$_2$, Ala, Val, Tyr, Phe)-Asp (Residues 41–53). After one step of Edman degradation, the composition of the residual peptide was: Lys, 1.00(1); *AEC, 0.10(0);* Asp, 1.90(2); Glu, 2.95(3); Gly, 2.11(2); Ala, 1.01(1); Val, 0.89(1); Tyr, 0.80(1); Phe, 0.92(1). Asp was found in 24% yield after hydrazinolysis. The composition of this peptide, together with the information of its NH_2- and COOH-terminal amino acid residues showed clearly that this fragment had to be in the COOH-terminal position of the protein and must overlap peptides T-5, T-1, T-6, C-4, and C-7. Since the sequence of this part of the molecule had been established, no further studies were done with peptide C-5.

Peptide C-7-Glu-Asp (Residues 52–53). One step of the Edman degradative reaction was carried out. PTH-Glu was directly identified. The residue when analyzed in the amino acid analyzer, with or without acid hydrolysis, indicated that aspartic acid was carboxyl terminal in this peptide.

The Stability of the Chelate Structure. Rubredoxin has been reported to be a markedly stable protein (Lovenberg and Sobel, 1965). However, the stability of the chelate structure has not been investigated in 50% ethanol, 8 M urea, or in guanidine hydrochloride solutions and was, therefore, studied. The results are shown in Figure 8.

Discussion

The sequence data derived from the tryptic and chymotryptic peptides made the placement of all fragments possible. Since the sole methionine residue of the protein was shown to be in the NH_2-terminal position of the protein, peptides T-2 and C-2 are the NH_2-terminal peptides. Peptide T-4 is the only fragment with a phenylalanine NH_2 terminal, which showed that it has to follow T-2 and overlap with C-2. Peptide C-3a gives an overlap between T-4 and T-3, and peptide C-3b overlaps fragments T-3 and T-5. Of the remaining two tryptic peptides, T-1 and T-6, peptide T-6 has to be COOH terminal since it does not contain Lys or AEC and has, in addition, the same COOH-terminal amino acid, aspartic acid, as the intact protein.

Edman degradation of T-5 showed that Ile is in the third position. Since only one Ile residue occurs in the protein the placement of C-8a, which has an Ile NH_2 terminal, after C-3b was assured. From the sequences of terminal groups of the chymotryptic peptides C-4, C-5, and C-7 and comparison with the tryptic fragments T-5, T-1, and T-6, it was obvious that C-7 has to be in the COOH-terminal position of the protein, preceded

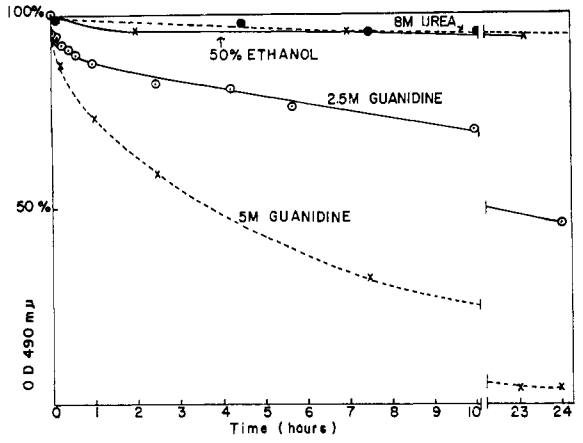

FIGURE 8: Stability of *M. aerogenes* rubredoxin at room temperature in 8 M urea (--O--), 50% ethanol (—X—), and 2.5 (—O—) and 5 M (--X--) guanidine hydrochloride. The protein concentration was 0.04 μmole/ml, and the solution contained 0.05 M Tris-HCl (pH 7.0) and 0.05 M NaCl.

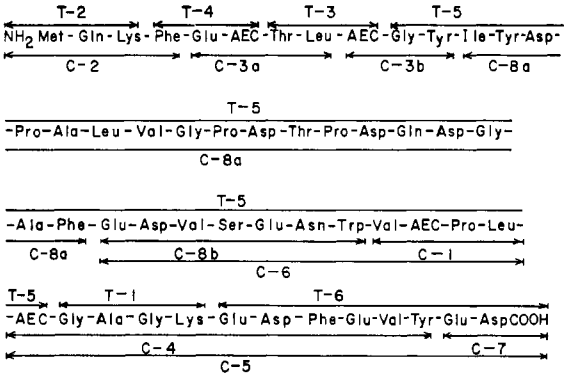

FIGURE 9: Construction of the amino acid sequence of the *M. aerogenes* rubredoxin. The placement of the tryptic and chymotryptic peptides are shown.

by C-4. Peptide C-5 is an overlapping peptide between C-4 and C-7. The position of peptide C-6 was determined from its component parts, C-1 and C-8b. Since C-8b and C-6 both have glutamic acid in the NH_2-terminal position, C-8b has to precede C-1 in the protein molecule. The complete sequence of *M. aerogenes* rubredoxin is shown in Figure 9.

Despite the fact that bacterial ferredoxins and rubredoxins have similar molecular weights and are functionally replaceable in many redox reactions (Lovenberg and Sobel, 1965), no similarity in the amino acid sequences of these two proteins can be found. Symmetry as observed in the ferredoxin sequences is absent in the case of rubredoxin.

The four cysteine residues (Bachmayer *et al.*, 1967a) occur in two sets of two residues within the molecule. Two cysteines are located in the NH_2-terminal part of the molecule (residues 6 and 9) and the other two near the carboxyl-terminal part (residues 38 and 41). In both sets, two other residues are spaced between the two cysteines.

Although rubredoxin contains no disulfide linkages, the single iron atom appears to act in the same manner as a disulfide bond in that it fixes and brings together closer the NH_2-terminal and COOH-terminal portions of the molecule (Bachmayer *et al.*, 1967a). Lovenberg (1966) and Gillard *et al.* (1965) have determined the optical rotatory dispersion spectra of ferredoxin and rubredoxin. In contrast to ferredoxin which had been shown to have low helical content, rubredoxin was reported to have a higher helical content.

Evidence that the four cysteine residues in rubredoxin participate in the binding of iron has been presented in a previous communication (Bachmayer *et al.*, 1967a). The type of electron paramagnetic spectrum observed points to the fact that the Fe(III) of rubredoxin is in a rhombic field. In addition to the four cysteine ligands, two more ligands are coordinated to the iron. Since rubredoxin lacks histidine and arginine, the following possible ligands have to be considered: the amino groups of the two lysines, the phenolic hydroxyl groups of the three tyrosine residues, the free COOH groups of the aspartic acid and glutamic acid residues, the indole of the tryptophan residue, and the free amino group of the methionine in NH_2-terminal position of the protein. In addition, the iron may possibly be coordinated to the *S* atom of methionine. The chelate structure is very stable to acid (Lovenberg and Sobel, 1965). In the present study, the chelate structure was shown to be stable to 8 M urea and 50% ethanol but was destroyed gradually by 5 M guanidine hydrochloride during a time interval of 24 hr. From the amino acid composition of the four rubredoxins reported thus far, the constancy of four cysteine residues and of three tyrosine residues is striking (Mayhew and Peel, 1966; Lovenberg, 1966). All rubredoxins thus far, contain at least two residues of lysine, but none of them contain arginine or histidine. The tryptophan content has remained constant at one residue per mole for rubredoxin from *M. aerogenes* (Bachmayer *et al.*, 1967a,b) and *P. elsdenii* (Mayhew and Peel, 1966) but two tryptophan residues have been found in *C. pasteurianum*, and *M. lactilyticus* rubredoxins (Lovenberg and Sobel, 1965; Lovenberg, 1966). The examination of the amino acid sequence of rubredoxins isolated from other species lacking one of the essential amino acids but still possessing activity and unchanged spectral properties would easily rule out some of the possible iron-binding ligands in the protein.

References

Bachmayer, H., Piette, L. H., Yasunobu, K. T., and Whiteley, H. R. (1967a), *Proc. Natl. Acad. Sci. U. S. 57*, 122.

Bachmayer, H., Yasunobu, K. T., and Whiteley, H. R. (1967b), *Biochem. Biophys. Res. Commun. 26*, 435.

Bachmayer, H., Whiteley, H. R., and Yasunobu, K. T. (1967c), *Pacific Slope Biochem. Conf. Davis, Calif.*

Bradbury, J. H. (1958), *Biochem. J. 68*, 475.

Fraenkel-Conrat, H., Harris, J. I., and Levy, A. L. (1955), *Methods Biochem. Anal. 2*, 388.

Gillard, R. D., McKenzie, E. D., Mason, R., Mayhew, S. G., Peel, J. L., and Stangroom, J. E. (1965), *Nature 208*, 769.

Goodwin, T. W., and Morton, R. A. (1946), *Biochem.*

J. 40, 628.
Keresztes-Nagy, S., and Margoliash, E. (1966), *J. Biol. Chem. 241*, 5955.
Konigsberg, W., and Hill, R. J. (1962), *J. Biol. Chem. 237*, 2547.
Le Gall, J., and Dragoni, N. (1966), *Biochem. Biophys. Res. Commun. 23*, 145.
Lovenberg, W. (1966), 14th Conference Protides of the Biological Fluids, Peeters, H., Ed., New York, N. Y., Elsevier.
Lovenberg, W., Sobel, B. E. (1965), *Proc. Natl. Acad. Sci. U. S. 54*, 193.
Mares-Guia, M., and Shaw, E. (1963), *Federation Proc. 22*, 528.
Mayhew, S. G., and Peel, J. L. (1966), *Biochem. J. 100*, 80P.
McConn, J. D., Tsuru, D., and Yasunobu, K. T. (1964), *J. Biol. Chem. 239*, 3706.
Moore, S. (1963), *J. Biol. Chem. 238*, 235.
Moore, S., and Stein, W. H. (1948), *J. Biol. Chem. 176*, 367.
Peterson, J. A., Basu, D., and Coon, M. J. (1966), *J. Biol. Chem. 241*, 5162.
Raftery, M. A., and Cole, R. D. (1963), *Biochem. Biophys. Res. Commun. 10*, 467.
Randerath, K. (1966), Thin Layer Chromatography, New York, N. Y., Academic.
Schroeder, W. A., Jones, R. T., Cormick, J., and McCalla, K. (1962), *Anal. Chem. 34*, 1570.
Smith, I., Ed. (1960), *in* Chromatographic and Electrophoretic Techniques, Vol. 1, New York, N. Y., Interscience.
Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.
Stadtman, T. C. (1965), Symposium Non-Heme Iron Proteins, Role in Energy Conversion, Yellow Springs. Ohio, Antioch, p 439.
Tanaka, M., Nakashima, T., Mower, H. F., Yasunobu, K. T. (1964), *Arch. Biochem. Biophys. 105*, 570.
Wang, S. S., and Carpenter, F. H. (1965), *J. Biol. Chem. 240*, 1619.
Witkop, G. (1961), *Advan. Protein Chem. 16*, 221.

Optical Properties of Actinomycin D. I. Influence of the Lactone Rings on Its Optical Activity*

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ABSTRACT: The optical activity of actinomycin D (AMD) as well as the ring-opened monolactone monocarboxylic acid and the dicarboxylic acid has been determined in several solvents between 580 and 190 mμ. The optical activity of the long-wavelength major absorption band (450–440 mμ) of AMD is weak. However, a strong optically active band is present at ~380 mμ. This band has been shown to require the presence of both lactone rings. Possible explanations for the optical activity of this band are discussed.

Several investigators have commented on the structural similarity of the chromophore actinocin of actinomycin D (AMD)[1] to the acridine dye, proflavine (Reich and Goldberg, 1964; Liersch and Hartmann, 1964, 1965; Cavalieri and Nemchin, 1964). In this context AMD may be considered, to a first approximation, as an optically inactive conjugated aromatic dye chemically bound at two sites to nonhelical but rigid cyclodepsipeptides. It would, therefore, be of some importance to determine if the absorption bands associated with the chromophore, which is insulated from the nearest asymmetric carbon atom by an amide group, are optically active and if any optical activity present is significantly affected by *internal* changes in the rigidity of depsipeptides as a result of chemical scission of either one or both lactone linkages. In this study the optical activity of the monocarboxylic (AMD monoacid) and dicarboxylic (AMD diacid) acid derivatives was measured and compared with that of AMD. Since a solvent can frequently change the position, magnitude, and sign of Cotton effects in an optical rotatory dispersion curve, such *external* factors as polarity and geometry of a solvent may conceivably alter the optical rotatory dispersion curve of AMD in changing the conformation of the molecule. Furthermore, since these perturbations may affect the interaction between AMD and DNA (Yamaoka and Ziffer, 1968), the optical rotatory dispersion, circular dichroism, and absorption spectrum of AMD were examined in three solvents (water, ethanol, and

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[1] Abbreviation used: AMD, actinomycin D.