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The amino acid sequence of rusticyanin isolated from *Thiobacillus ferrooxidans*

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The amino acid sequence of rusticyanin, a copper protein, purified from the iron-oxidizing bacterium *Thiobacillus ferrooxidans* was determined. Rusticyanin contained 154 amino acid residues in a single polypeptide chain and its molecular weight was calculated to be about 16400 based on the amino acid sequence. The N-terminal sequence up to the 20th residue of the protein apparently resembled those of *Methylobacterium extorquens* AM1 amicyanin and poplar leaf plastocyanin rather than those of azurin family proteins. In the C-terminal region of the sequence, rusticyanin had one cysteine, one histidine and one methionine which are conserved through many copper proteins. In the middle region of the sequence, rusticyanin was not similar to any other copper protein. The sequence nearby His^{84} of rusticyanin was similar to those of other copper proteins to some extent. However, Asn which follows His^{84} and is highly conserved in other copper proteins did not exist in rusticyanin. Therefore, it seemed difficult to conclude on the basis of the results obtained in the present study that His^{84} in rusticyanin was the fourth ligand to the copper atom.

Thiobacillus ferrooxidans; Amino acid sequence; Rusticyanin; Copper protein

1. INTRODUCTION

Thiobacillus ferrooxidans is an acidophilic chemolithoautotroph which obtains energy for growth by the oxidation of ferrous iron to ferric iron at pH 2.0. The electron transport chain of the bacterium has been investigated by some researchers. Cobley and Haddock [1] have shown that rusticyanin is directly reduced by ferrous iron in the presence of a cell-free extract prepared from *T. ferrooxidans*, and Cox and Boxer [2] have indicated that rusticyanin may function as the primary electron acceptor for ferrous iron in the bacterial electron transport chain. However, Fukumori et al. [3] have found that an iron-sulphur protein with M_r of 63 000 purified from *T. ferrooxidans* reduces cytochrome *c*-552 of the bacterium but not rusticyanin with ferrous iron at pH 3.0. They have called the iron-sulphur protein Fe(II)-cytochrome *c*-552 oxidoreductase. Kai et al. [4] have purified an *a*-type cytochrome oxidase from the bacterium and found that the oxidase oxidizes at pH 3.0 the reduced form of rusticyanin as well as ferrocytochrome *c*-552. In addition, rusticyanin functions as an electron carrier from cytochrome *c*-552 to the *a*-type oxidase. Namely, rusticyanin is a copper protein which is stable at pH 3.0 and reacts with the *a*-type oxidase. The properties of rusticyanin prompted us to determine its amino acid sequence. In the present study, we determined the amino acid sequence of *T. ferrooxidans* rusticyanin and compared the sequence with those of several copper proteins.

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2. MATERIALS AND METHODS

2.1. *Microorganism and cultivation*

Thiobacillus ferrooxidans was kindly supplied to us by Drs H. Shiota and N. Wakao (Iwate University, Morioka, Japan) [5]. The bacterium was cultivated at pH 2.0 in 9K medium of Silverman and Lundgren [6] supplemented with $FeSO_4 \cdot 7H_2O$ at the concentration of 100 g/l. The pH was adjusted to 2.0 with conc. H_2SO_4. The large scale cultivation of the organism was performed as previously described [7].

2.2. *Purification of rusticyanin*

Rusticyanin was purified to an electrophoretically homogeneous state by the following procedure. The cells suspended in deionized water were treated 3 times with a sonic oscillator (20 kHz, 250 W) for 15 min and then treated with a French pressure cell at 1200 kg/cm^2. The suspension thus treated was centrifuged at 3000 × *g* for 10 min to remove unbroken cells. Solid $(NH_4)_2SO_4$ was added to the resulting supernatant to 10% saturation and the supernatant thus treated was centrifuged at 144 000 × *g* for 1 h. The resulting supernatant was dialyzed against 10 mM sodium phosphate buffer, pH 6.0. The dialyzed solution of rusticyanin was applied to a CM-cellulose column (3 × 25 cm) which had been equilibrated with the same buffer as used for the above dialysis. Rusticyanin adsorbed on the column was eluted with a linear gradient produced by 200 ml each of 10 mM sodium phosphate buffer, pH 6.0, and the buffer containing 0.5 M NaCl. The eluates containing rusticyanin were combined and dialyzed against 10 mM sodium phosphate buffer, pH 6.0. The dialyzed solution was subjected to a CM-Toyopearl column (3 × 10 cm) and the protein adsorbed on the column was eluted with a linear gradient of NaCl (0–0.2 M). The eluates which contained rusticyanin were combined, dialyzed

against 5 mM sodium phosphate buffer, pH 6.0, and charged on a hydroxylapatite column (3 × 10 cm) which had been equilibrated with the same buffer as used for the dialysis. Rusticyanin adsorbed on the column was eluted with a linear gradient of sodium phosphate buffer, pH 6.0 (5–300 mM). The fraction containing rusticyanin was concentrated with a CM-cellulose column (1 × 5 cm) and then applied to gel filtration with a Sephadex G-75 column (2.5 × 110 cm) which had been equilibrated with 10 mM sodium phosphate buffer, pH 6.0, containing 0.25 M NaCl. The eluates which contained rusticyanin were collected, combined, and used for the amino acid sequencing. The purity of rusticyanin was checked by the absorbance ratio of A_{278}/A_{597} and polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. The ratio of pure rusticyanin was assumed to be 2.3 [2]. Carboxymethyl (Cm)-rusticyanin was prepared by the method of Crestfield et al. [8].

2.3. *Amino acid analysis*

Amino acid compositions of Cm-rusticyanin and peptides were determined by using an automated amino acid analyzer (Irica Instruments, Inc., Model A-3300, Kyoto, Japan) with an acceleration system according to the methods of Speckman et al. [9] after the samples had been hydrolyzed with 6 M HCl for 24 h at 110°C in an evacuated sealed tube.

2.4. *Amino acid sequence determination*

Cm-rusticyanin was digested by the following three methods: (i) 500 μg of Cm-rusticyanin was digested in 0.2 M ammonium phosphate buffer, pH 8.0, with 5 μg of TPCK-trypsin for 8 h at 37°C, (ii) 500 μg of Cm-rusticyanin was digested in 50 mM sodium phosphate buffer, pH 7.8, with 20 μg of *Staphylococcus* V8-protease for 24 h at 37°C, and (iii) 500 μg of Cm-protein was chemically cleaved in 0.1 H HCl with 20 mg of cyanogen bromide for 48 h at 37°C. After each digestion, peptides produced were separated by reversed-phase HPLC on a COSMOSIL $5C_{18}$ column (4.6 × 250 mm) in 0.1% (v/v) trifluoroacetic acid with a linear gradient of acetonitrile from 0 to 70% (v/v) at 25°C. Some peptides were further purified by rechromatography using the reversed-phase HPLC column under different conditions. The N-terminal sequences of rusticyanin and the peptides purified were determined by a gas-phase protein sequencer (Applied Biosystems, model 470A, USA).

2.5. *Reagents*

TPCK-Trypsin (EC 3.4.21.4) and *Staphylococcus* V_8-protease (EC 3.4.21.19) were purchased from Worthington Biochemical Company (USA) and Sigma Chemical Company (USA), respectively. CM-cellulose and Sephadex G-75 were purchased from Pharmacia (Sweden) and CM-Toyopearl from Tohso Corporation (Tokyo, Japan). Hydroxylapatite was prepared by the methods of Tiselius et al. [10]. A COSMOSIL $5C_{18}$ column was purchased from Nacalai tesque (Kyoto, Japan). All other chemicals were of the highest grade commercially available.

3. RESULTS

By the gas-phase Edman degradation, N-terminal sequence of rusticyanin was determined to be Gly-Ala-Leu-Asp-Ser-Ser-Trp-Lys-Glu-Ala-Thr-Leu-Pro-Glu-Val-Lys-Ala-Met-Leu-Gln-Lys-. The amino acid analysis and sequence analysis of rusticyanin showed that one Cys exists in a molecule. The results showed that no disulfide bond occurred in the rusticyanin molecule unlike the case of azurins. Carboxypeptidase B released lysine from rusticyanin after a 30 min incubation. This result suggested the C-terminal residue of rusticyanin to be Lys. The tryptic digests and V8-protease digests were separated into each peptide with HPLC. Nonspecific cleavages by the proteases were not detected. The chemical cleavage of rusticyanin with cyanogen bromide was incomplete. Therefore, some peptides thus obtained needed further purification by HPLC before being applied to the gas-phase Edman degradations. The result obtained from the sequence study of rusticyanin is summarized in Fig. 1. Rusticyanin contained 154 amino acid residues in a single polypeptide chain and had the amino acid composition: Cys, 1; Asp, 8; Asn, 4; Thr, 14; Ser, 8; Glu, 4; Gln, 2; Pro, 14; Gly, 17;

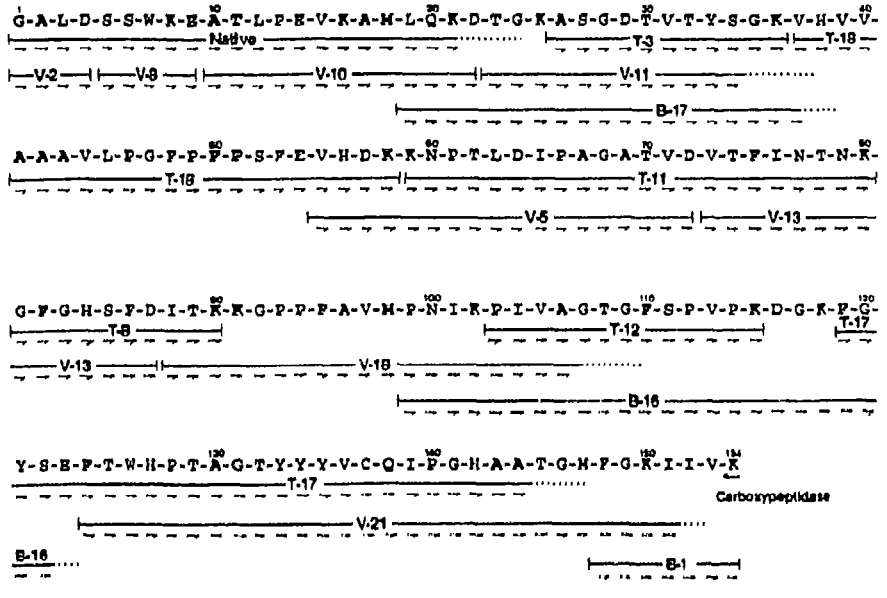

Fig. 1. Primary structure of rusticyanin from *T. ferrooxidans*. Peptides derived from the protein by digestion with trypsin (T), by digestion with *Staphyrococcus* V8-protease (V) and by digestion with cyanogen bromide (B) are respectively shown under the sequences. —— indicates amino acid residues determined by a gas-phase protein sequencer. Residue released by the carboxypeptidase B is shown by ·—. Cys was determined as Cm-cysteine.

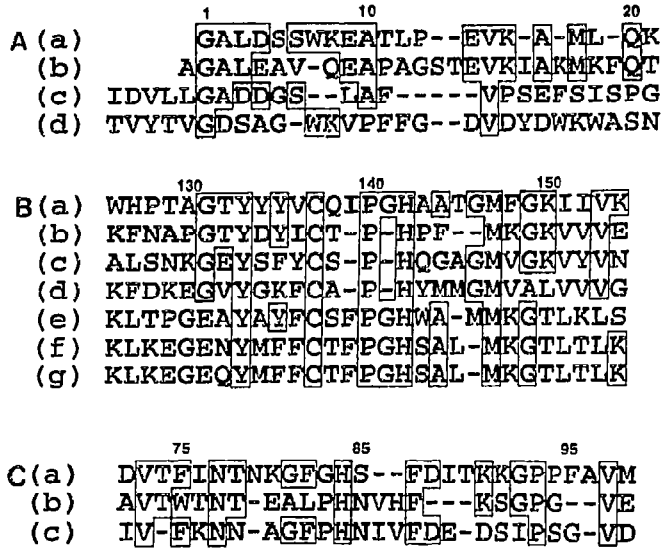

Fig. 2. Comparison of the amino acid sequence of *T. ferrooxidans* rusticyanin with those of several blue copper proteins. (A) Amino acid sequence alignments in the N-terminal region. (B) Amino acid sequence alignments in the C-terminal region. (C) Amino acid sequence alignments around His^{84}. (a) *T. ferrooxidans* rusticyanin, (b) *M. extorquens* AM1 amicyanin [16], (c) poplar leaf plastocyanin [16], (d) *M. extorquens* AM1 azurin [16], (e) *Paracoccus denitrificans* azurin [11], (f) *Pseudomonas fluorescens* azurin [12], and (g) *Ps. aeruginosa* azurin [13]. The amino acid residues are boxed which are identical with those of *T. ferrooxidans* rusticyanin. Residues are numbered by rusticyanin numbering.

Ala, 14; Val, 14; Met, 3; Ile, 8; Leu, 5; Tyr, 5; Phe, 11; Lys, 15; His, 5; Trp, 2. Rusticyanin lacked the arginine residue as reported previously [2]. The molecular weight of the protein was calculated to be about 16 400 based on the amino acid sequence.

4. DISCUSSION

The amino acid sequence of rusticyanin determined in the present study shows unique features, when the sequence is compared with those of other copper proteins which have been sequenced (azurin [11–13], plastocyanin [14], stellacyanin [15] and amicyanin [16]). No disulfide bond in the molecule occurs in the N-terminal region of rusticyanin. Recently, *Thiobacillus verstus* amicyanin has been found to have only one Cys [17]. The sequence in the N-terminal region of the protein is more similar to those of *Methylobacterium extorquens* AM1 amicyanin [15] and poplar leaf plastocyanin [16] (Fig. 2A) than those of other copper proteins so far sequenced [11–14,16]. However, the middle region of the sequence of rusticyanin is not homologous to those of any other copper proteins. On the basis of the three-dimensional structures of azurin and plastocyanin [14,18–21], it is known that 4 amino acid residues ligate to a copper atom in these proteins. Three of the residues ligating to the copper atom are Cys_{137}, His_{142} and Met_{147}, and these residues gather in the C-terminal region. Fig. 2B shows the C-terminal sequence alignments of several copper proteins. The relative positions of 3 ligands to the copper atom are the same among the blue copper proteins compared. *T. ferrooxidans* rusticyanin possesses Cys_{137}, His_{142} and Met_{147} at the positions apparently homologous to the respective positions of other copper proteins. The similarity of rusticyanin to several other copper proteins observed in the C-terminal sequence alignments will suggest that the C-terminal region of rusticyanin may form the same spatial structure as those plastocyanin. In addition, the structural similarity of rusticyanin to azurin in the C-terminal region is predicted also by using Chou-Fasman method [22] (data not shown).

In general, the forth ligand to the copper atom, His, lies in the region of a highly conserved sequence and is followed by an invariant Asn in all blue copper proteins so far sequenced: -Gly-His-Asn- for azurin [11–13], -Pro-His-Asn- for plastocyanin [14] and -Phe-His-Asn- for stellacyanin [15]. The Asn which is present in the position corresponding to the above sequences and follows His^{84} is replaced by Ser in rusticyanin. However, the amino acid sequence of rusticyanin around the His^{84} is relatively similar to the sequences around the His mentioned above in amicyanin and plastocyanin (Fig. 2C). Nevertheless, it seems difficult to conclude from this similarity whether His^{84} ligates to copper atom in the molecule. There is a possibility that the fourth ligand to copper may be an amino acid residue other than His, i.e. it may be Met or Asp. The copper binding site in rusticyanin may be different from that in other copper proteins and the fourth ligand may be responsible for the unusual properties of rusticyanin such as having extremely high redox potential (+680 mV at pH 3.2) [2] and stability under acidic conditions [4]. Further investigations are necessary to decide the amino acid residue which ligates to the copper atom as the fourth ligand.

T. ferrooxidans rusticyanin as well as cytochrome *c*-552 functions as the direct electron donor for *a*-type cytochrome *c* oxidase of the bacterium [4]. Therefore, it seems that both rusticyanin and cytochrome *c*-552 have structurally similar binding sites for the oxidase. However, further information about the structures of cytochrome *c*-552 as well as rusticyanin is needed to discuss the interaction with the oxidase of these proteins. Determination of the amino acid sequence of cytochrome *c*-552 is now in progress in our laboratory.

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