MYCOBACTERIUM SMEGMATIS FERREDOXIN: A UNIQUE DISTRIBUTION OF CYSTEINE RESIDUES CONSTRUCTING IRON–SULFUR CLUSTERS

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1. Introduction

Some bacteria contain 8 Fe–8 S ferredoxin with two (4 Fe–4 S) clusters of widely different midpoint potentials (~700 mV apart). These ferredoxins are *Azotobacter vinelandii* ferredoxin I [1], *Rhodospirillum rubrum* ferredoxin IV [2] and *Mycobacterium flavum* ferredoxin I [3]. EPR analyses [2–4] have shown that these ferredoxins were paramagnetic (g=2.01) in oxidized state and diamagnetic in reduced state, contrary to 8 Fe–8 S ferredoxins from *Clostridium* and *Chromatium* which were paramagnetic (g=1.94) in reduced state. This evidence shows that the two clusters function between the same pair of oxidative states (–2 and –1 oxidation states) as the single (4 Fe–4 S) cluster in *Chromatium* high-potential iron protein (HIPIP) [4].

The amino(N)-terminal sequence of *A. vinelandii* ferredoxin I was investigated [5] and it was concluded that an unusual nature of the two clusters correlated to the different distribution of cysteine residues from that of *Peptococcus* ferredoxin [6]. We have reported [7] the complete amino acid sequence of *Pseudomonas ovalis* ferredoxin I which had a similar magnetic nature to that of *A. vinelandii* ferredoxin I and shown that the sequence was quite homologous with that of *A. vinelandii* ferredoxin I, but there was no substantial difference in the distribution of 8 cysteine residues between *P. ovalis* ferredoxin I and *Peptococcus* [6] or *Chromatium* [8,9] ferredoxin.

A 8 Fe–8 S ferredoxin with mol. wt 12 800 has been isolated from *Mycobacterium smegmatis* Takeo [10] having similar properties to those of *A. vinelandii* ferredoxin above. This ferredoxin contained 8 cysteine residues which must fulfill the minimum requirement for constituting the two (4 Fe–4 S) clusters. To clarify the still ambiguous nature of the distribution of cysteine residues necessary for the iron chelation in the ferredoxins from *A. vinelandii* and *P. ovalis*, we studied the sequence of *M. smegmatis* ferredoxin.

This paper describes the complete amino acid sequence of *M. smegmatis* ferredoxin and discusses the structural relationship between these ferredoxins.

2. Materials and methods

M. smegmatis ferredoxin was prepared as in [10]. After precipitation of ferredoxin with trichloroacetic acid the apoprotein was reduced with 2-mercaptoethanol and carboxymethylated with iodoacetic acid [11]. The amino acid composition of carboxymethyl-(Cm)-ferredoxin was determined as usual after 6 N HCl hydrolysis for 24 h and 72 h. Tryptophan was analyzed after hydrolysis with 3 N mercaptoethanesulphonic acid [12].

Cm-ferredoxin (1.8 μmol) was digested with trypsin. The digest was fractionated by gel filtration on a Bio-Gel P-10 column (2 × 197 cm) equilibrated with 0.2 M ammonium bicarbonate and eluted with the same buffer. Cm-ferredoxin was separately digested with staphylococcal protease and the peptides produced were separated on the same column as above. Some peptide fractions were further purified by paper electrophoresis at pH 3.6 or pH 6.5.

The N- and carboxyl(C)-terminal sequences of the Cm-ferredoxin and peptides were determined by a

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manual Edman degradation method and carboxypeptidase method, respectively [13,14]. Some tryptic peptides containing a lysine residue at the C-terminus were coupled to aminopolystyrene of aminopropyl glass using *p*-phenylenediisothiocyanate (DITC) [15] and sequenced with the solid phase Edman degradation using an LKB 4020 solid-phase peptide sequencer, Sweden. Other detailed procedures of sequence studies were essentially the same as in [16,17].

3. Results and discussion

3.1. *Sequence studies of* M. Smegmatis *ferredoxin*

The amino acid composition of Cm-ferredoxin of *M. smegmatis* was:

Lys, 3.94 (4); His, 0.93 (1); Arg, 1.01 (1); Cm-Cys, 8.27 (8); Asp, 14.9 (15); Thr, 2.02 (2); Ser, 4.13 (4); Glu, 15.2 (15); Pro, 10.2 (10); Gly, 7.13 (7); Ala, 10.7 (11); Val, 9.28 (9); Met, 0.90 (1); Ile, 5.36 (6); Leu, 2.87 (3); Tyr, 6.24 (6); Phe, 2.00 (2); Trp, 0.99 (1).

The numbers in parentheses were deduced from the established sequence. A manual Edman degradation of Cm-ferredoxin revealed the N-terminal sequence up to 15 residues to be:

Thr–Tyr–Val–Ile–Ala–Glu–Pro–Cys–Val–Asp–Val–Lys–Asp–Lys–Ala–

Carboxypeptidase Y released only aspartic acid from Cm-ferredoxin.

Five peptides, T-1–T-5, and 8 peptides, S-1–S-8, were isolated from tryptic and staphylococcal protease digests, respectively. Figure 1 shows the summary of sequence studies of these peptides. Peptides T-5, S-1–S-4, and S-6 were completely sequenced by manual Edman degradation. Peptides T-1 and T-4, which were relatively small peptides, were degraded successfully to step 8 after which PTH-amino acids were hardly detectable on a thin layer. Thus, these peptides were degraded using a solid phase sequencer after attaching to resin or glass with DITC procedure and all PTH-amino acids except for the first and the last (12th) ones were identified. The C-terminal residues were assumed to be lysine which remained attached to the support. The C-terminal sequences of peptides T-2 and T-4 were deduced by digestions with carboxypeptidase B followed by digestion with carboxypeptidase A and those of peptides S-5 and S-7 by digestion with carboxypeptidase Y. Peptide T-2 was further digested with thermolysin and 4 peptides, T-2–Th-1 to T-2–Th-4, were isolated by paper electrophoresis at pH 3.6. The alignment of these peptides is shown in fig.1 and the partial sequence of original peptide T-2 was confirmed. Peptide T-3 was further digested with chymotrypsin and the digest was fractionated on a Bio-Gel P-4 column. Five peptides, T-3–C-1 to T-3–C-5, were obtained. Each of chymotryptic peptides was sequenced by manual Edman degradations. Carboxypeptidase Y digestions of Peptides T-3–C-2 and T-3–C-5 determined their C-terminal sequences. Carboxypeptidase A released tryptophan from Peptide T-3–C-3, showing that the ambiguous residue at step 7 of Edman degradation of peptide S-7 was tryptophan.

The N-terminal sequence of Cm-ferredoxin gave the overlaps of peptide T-1–T-2 and peptide S-1–S-3. The sequences of peptides S-5–S-8 gave the complete alignment for chymotryptic peptides of peptide T-3 and overlap of peptide T-2–T-4. Peptide T-5 was the C-terminal peptide, being judged from its C-terminal residue. These results aligned all peptides in order establishing the complete amino acid sequence of *M. smegmatis* ferredoxin.

3.2. *Comparison of sequences of* M. smegmatis *ferredoxin I and* P. ovalis *ferredoxin and distribution of cysteine residues*

It has been reported that *A. vinelandii* ferredoxin I [4], *R. rubrum* ferredoxin IV [2] and *M. flavum* ferredoxin I [3] have two (4 Fe–4 S) clusters operating between the –2 and –1 oxidation states as in *Chromatium* HIPIP, but the midpoint potentials of the two clusters were ~700 mV different. These ferredoxins have common characteristics in molecular weight, amino acid composition, absorption spectrum and EPR signal. *P. ovalis* ferredoxin I [18] and *M. smegmatis* ferredoxin [10] showed the EPR signal characteristic to HIPIP-type cluster and had absorption spectra and amino acid compositions similar to those of *A. vinelandii* ferredoxin I. Therefore, these two ferredoxins seem to belong to the same class of ferredoxin as above.

M. smegmatis ferredoxin was composed of 106

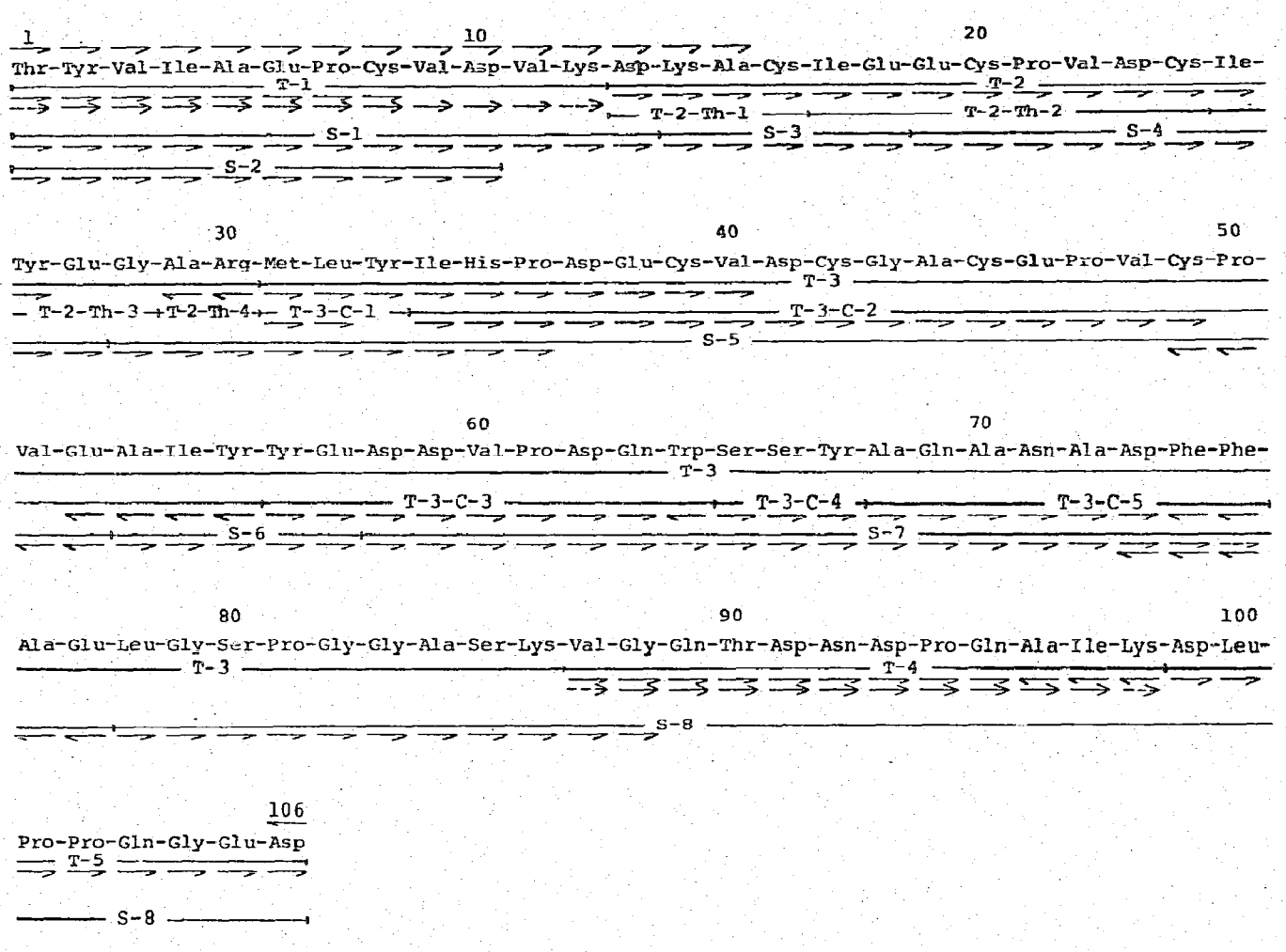

Fig.1. Summary of the sequence studies of *M. smegmatis* ferredoxin. T-, S-, C- and Th- refer, respectively, to peptides derived by tryptic and staphylococcal protease digestion of Cm-ferredoxin, and by chymotryptic digestion of peptide T-3 and thermolysin digestion of peptide T-2. Arrows (→) and (←) above the sequence show, respectively, Edman degradation and carboxypeptidase Y digestion on Cm-ferredoxin. Arrows (→) and (←) below the sequences of peptides show, respectively, Edman degradations and carboxypeptidase digestion on peptides. Arrows (↔) below the peptides, T-1 and T-4, show Edman degradations by a solid phase procedure. Dotted arrows represent ambiguous identification.

amino acid residues with 8 cysteine residues and *P. ovalis* ferredoxin the same number of residues with 9 cysteine residues [7]. Comparison of these two ferredoxin sequences as shown in fig.2 gave an interesting feature; 37 residues were identical between the two sequences and most of these residues were located at the N-terminal half (55% and 15% were identical in the N- and C-terminal halves, respectively). We showed [7] that the N-terminal sequence of *P. ovalis* ferredoxin I was quite similar to that of *A. vinelandii* ferredoxin I, although only partial sequence of the latter was available and all cysteine residues were located at the corresponding positions in *P. aerogenes* ferredoxin, suggesting that homologous distribution of cysteine residues must be important to constitute the two (4 Fe–4 S) clusters [7].

The chelate structures of two (4 Fe–4 S) clusters of *P. aerogenes* ferredoxin [19] and a (4 Fe–4 S) cluster of *Chromatium* HIPIP [20] are known. *P. ovalis* ferredoxin I had 9 cysteine residues at positions 8, 11, 16, 20, 24, 39, 42, 45 and 49 and 8 residues except one at position 24 were located at the

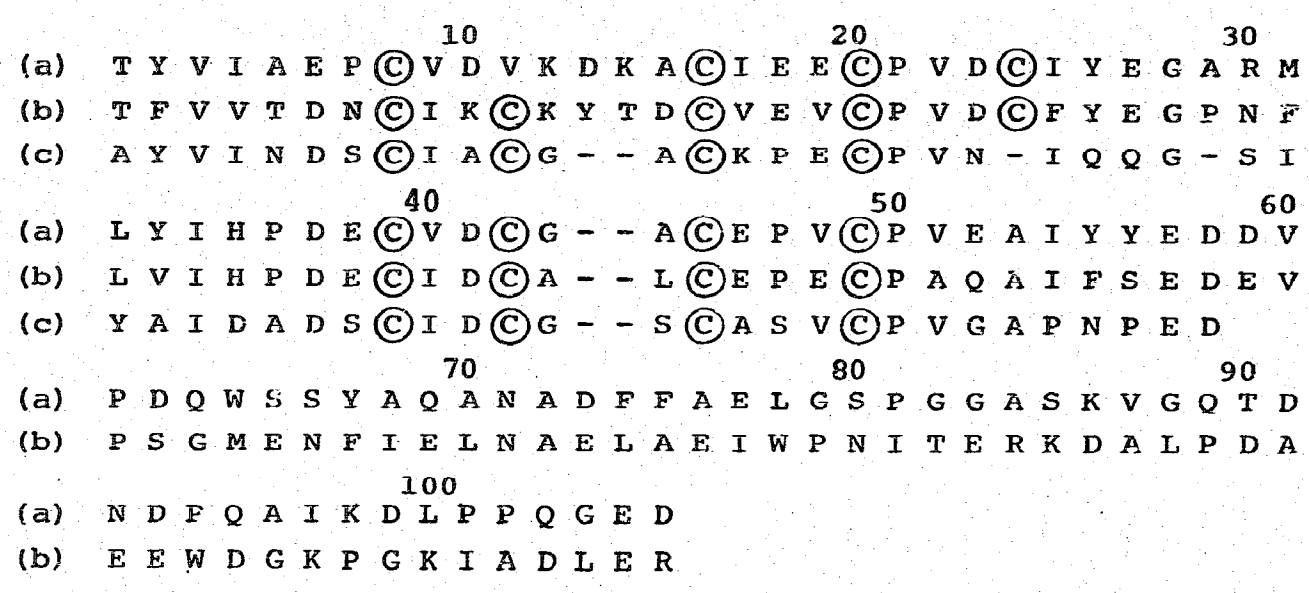

Fig.2. Comparison of ferredoxins from *M. smegmatis* (a), *P. ovalis* (b) and *P. aerogenes* (c). The numbering is for *M. smegmatis* ferredoxin. Cysteine residues are circled for emphasis, and – refers to a gap to make all alignments most probable.

same positions as those found in *P. aerogenes* ferredoxin [7] and there was no substantial difference in cysteine distribution between *P. ovalis* ferredoxin I and other bacterial ferredoxins which had two low potential (4 Fe–4 S) clusters. *M. smegmatis* ferredoxin, however, had only 8 cysteine residues and a notable change in cysteine distribution was found in comparison with *P. ovalis* ferredoxin I. They are located at positions 8, 16, 20, 24, 39, 42, 45, and 49 and the residue at position 11 was substituted to valine in *M. smegmatis* ferredoxin. We may, therefore, conclude that the 8 cysteine residues required for the formation of two clusters are at positions 8, 16, 20, 24, 39, 42, 45, and 49 in both the ferredoxins from *P. ovalis* and *M. smegmatis*; the previous speculation for chelate structure in *P. ovalis* ferredoxin [7] should be withdrawn on the occasion of the new finding described here.

This conclusion may be in conflict with the fact that the cysteine residues found in the N-terminal sequence of *A. vinelandii* ferredoxin I were in different positions from those in *M. smegmatis* ferredoxin. However, *Azotobacter* ferredoxin was reported to have 8 cysteine residues, 7 of which were located at positions 8, 11, 16, 20, 24, 39, and 42, but the 8th cysteine residue was not determined and the residue at position 45 was ambiguously identified [5]. Therefore, an additional sequence study of *A. vinelandii* ferredoxin I is required to confirm our proposal.

It is hard to speculate for the present which 4 cysteine residues participate in the constitution of one cluster and the other, although residues at positions 20, 39, 42, and 45 are located at the homologous position with those constituting one cluster of *P. aerogenes* ferredoxin. *M. flavum* ferredoxin II with 4 cysteine residues had only one HIPIP-type cluster of midpoint potential at ~–420 mV [3]. Therefore, the sequence of this ferredoxin may give some insight to our proposal.

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