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Primary structure of protein B from *Pseudomonas putida*, member of a new class of 2Fe-2S ferredoxins

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The primary structure of the 2Fe-2S ferredoxin (protein B) from the benzene dioxygenase system of *Pseudomonas putida* strain NCIB 12190 was determined by gas-phase sequencing of the protein and its fragments. Fast atom bombardment mass spectrometry indicated a molecular mass of 11860 Da. The sequence contained five cysteine residues, four of which would be required to coordinate the iron-sulphur cluster. The amino acid sequence determined in the present study is compared to that of a protein deduced from the DNA sequence from another strain of *Pseudomonas putida*. Little sequence homology was observed when protein B was compared to 2Fe-2S ferredoxins from plant and cyanobacterial sources. The novel sequence determined here suggests a new class of ferredoxin, which is consistent with the observed mid-point redox potential being significantly less negative (−155 mV) than those of the 2Fe-2S ferredoxins involved in photosynthesis (−310 to −455 mV)

Ferredoxin; Protein sequence; Benzene dioxygenase; Iron-sulfur protein

1. INTRODUCTION

Pseudomonas putida has been shown to use benzene as its sole source of carbon, converting benzene to *cis*-benzene glycol [1]. The bacterium has been shown to contain a soluble enzyme system which converts benzene to *cis*-benzene glycol, in a system dependent upon NADH and Fe^{2+}. Similar systems have been identified for the oxidation of toluene [2], isopropyl benzene [3] and pyrazon (5-amino-4-chloro-2-*N*-pyridazin-3-one) [4]. The benzene dioxygenase system was shown to consist of a flavoprotein A2 (81 kDa), an iron-sulphur protein B (12.3 kDa) and the terminal dioxygenase A1 (215 kDa) [5]. On the basis that its function is simple electron transfer, it was described as a ferredoxin [6]. Its midpoint redox potential, −155 mV, is less negative than the well-studied [2Fe-2S] ferredoxins involved in plant and cyanobacterial photosynthesis (−310 to −455 mV) [7].

This paper describes the determination of the amino acid sequence of the ferredoxin from *Ps. putida* strain ML2 (NCIB 12190). The DNA sequence of the genes cloning for the benzene dioxygenase system from another strain of *Ps. putida* (isolate BE-81) has just been published [8]. The amino acid and DNA sequences are in complete agreement with the exception of four conservative and one serine to proline substitution, reflecting the difference in source material. One major difference is that the protein sequence in the present study, from strain NCIB 12190, is fifteen residues

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Abbreviations: PEC, pyridylethyl cysteine derivative; PTH, phenylthiohydantoin derivative; PTC, phenylthiocarbamyl derivative; FAB, fast atom bombardment; NCIB, National Collection of Industrial Bacteria

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longer at the N-terminus compared to that deduced from the DNA sequence of strain BE-81.

These studies reveal a novel organization of the iron-sulphur cluster-binding region, and the presence of an adjacent histidine, which may explain the unusually high redox potential of the protein.

The plant-type [2Fe-2S] ferredoxins from many species have been extensively studied [9]. Structurally these proteins are very similar with three of the cysteines involved in coordinating the [2Fe-2S] cluster located in the following highly conserved region,

39				44		47		
Ser	Cys	Arg	Ala	Gly	Ser	Cys	Ser	Ser	Cys.
		Gln			Ala		Thr		

Other [2Fe-2S] ferredoxins are involved in electron transfer to cytochrome P450-containing monooxygenase systems in mitochondria such as those from adrenal glands (adrenodoxin) and in a camphor hydroxylase of another strain of *Ps. putida* (putidaredoxin). These ferredoxins show a more axial type of EPR signal but the amino acid sequence reveals a similar arrangement of cysteines [10].

A [2Fe-2S] ferredoxin from *Clostridium pasteurianum* with unusual spectroscopic properties was reported to have little structural homology with the chloroplast type ferredoxins and the position of the cysteines was substantially different from the previously reported ferredoxin sequences [11].

2. MATERIALS AND METHODS

Organism: *Ps. putida* strain ML2, deposited as NCIB 12190. *Staphylococcus aureus* V8 proteinase, carboxypeptidase Y and dithiothreitol were obtained from Boehringer. TPCK-trypsin and TLCK treated a-chymotrypsin were from Worthington. The lysine-specific proteinase from the fungus *Armillaria mellea* was a generous gift from Professor S. Doonan (University of Cork, Eire). Trifluoroacetic acid was purchased from Pierce. All sequencing reagents were from Applied Biosystems. Hydrochloric acid, formic acid and guanidinium chloride (Aristar grade) were obtained from BDH. Iodo-[^{14}C]acetic acid was from Amersham. 4-Vinylpyridine was obtained from Aldrich and redistilled before use.

2.1. *Preparation of protein B for sequence analysis*

Protein B was isolated from *Ps. putida* cultures grown with benzene as a sole source of carbon as reported [5].

The Apo-ferredoxin was prepared by the method of Meyer et al. [11] as follows: the ferredoxin was dissolved in 50 mM Tris-HCl, pH 8, precipitated with 0.5 M HCl with stirring at 0°C for 30 min and centrifuged at 10000 × *g* for 10 min. The pellet was resuspended in 100 mM Tris-HCl, pH 8.0, and reprecipitated as before. The protein was dissolved in 50 mM *N*-ethylmorpholine acetate, pH 8.0, with 6 M guanidinium chloride and the cysteines reduced with either 10 mM β-mercaptoethanol or 5 mM dithiothreitol for 1 h at 25°C. The protein was then carboxymethylated with iodoacetic acid (20-fold molar excess) or pyridethylated [12] with 4-vinylpyridine for 3 h at 25°C.

2.2. *Generation of peptides*

2.2.1. Lysine-specific proteinase digestion

Carboxymethylated-protein B was cleaved specifically at the N-terminal side of lysine residues with *A. mellea* proteinase [13]. 200 μg protein B was dissolved in 0.5 ml *N*-ethylmorpholine acetate buffer (0.1 M, pH 7.5) and digested with lysine-specific proteinase for 2 h at 37°C at an enzyme/substrate ratio of 1:100. A further aliquot of the proteinase was added to reduce the ratio to 1:50 and digestion continued for a further 2 h at 37°C.

2.2.2. Arginine-specific cleavage

Carboxymethylated-protein B (20 μg) was dissolved in 0.1 M Na_2CO_3, pH 9, and reacted with citraconic anhydride (6 × 2 μl aliquots) with continual stirring for 1 h. The pH was maintained with additions of solid Na_2CO_3. The protein was desalted by HPLC gel-exclusion chromatography in 0.1 M *N*-ethylmorpholine acetate buffer, pH 7.5. The protein was digested with trypsin at a 1:40 weight ratio for 20 h at 37°C.

2.2.3. Chymotrypsin digestion

PEC-protein B (30 μg) was dissolved in 0.2 ml *N*-ethylmorpholine acetate buffer (0.1 M, pH 8) and chymotrypsin added to a weight ratio of 1:40. After incubation for 2 h at 37°C, a further addition of the proteinase was made to yield a weight ratio of 1:20. The incubation was continued for a further 12 h and then stopped by lyophilisation.

2.2.4. Digestion with *S. aureus* V8 proteinase

PEC-protein B (20 μg) was digested with 0.4 μg V8 proteinase for 2 h at 37°C in 0.1 M *N*-ethylmorpholine acetate buffer, pH 7.5. Further proteinase was added to a final weight ratio of 1:25 and the incubation continued for a total of 16 h.

2.2.5. Digestion with trypsin

Carboxymethylated-protein B (24 μg) was digested with trypsin (1:30 proteinase:substrate) in 0.1 M *N*-ethylmorpholine acetate buffer, pH 7.5, for a total of 14 h at 37°C.

2.2.6. Cleavage with cyanogen bromide

Carboxymethylated protein B (50 μg) was dissolved in 0.5 ml of 40% aqueous formic acid and cyanogen bromide added (100-fold excess over methionine residues). The sample was flushed with nitrogen and incubated for 26 h at 4°C in the dark. The reaction was terminated by diluting to 5% aqueous formic acid and peptides were lyophilised. Under these conditions the large extent of cleavage at both of the tryptophan residues was unexpected.

2.3. *Purification of peptides by HPLC*

Peptides were purified using a Gilson HPLC system fitted with a Gilson 116 UV detector. Peptides were monitored at 215 and 280 nm, with pyridethylated peptides detected at 254 nm. Reverse-phase chromatography was performed with Bakerbond C_4 (250 × 4.6 mm) and Brownlee RP-300 C_8 (220 × 2.1 mm) columns. Gradient elution with water/acetonitrile containing 0.1% (v/v) trifluoroacetic acid was performed typically from 0 to 60% acetonitrile at 1% increase min^{-1}. Gel-exclusion HPLC was carried out with a TSK 2000SW (300 × 7 mm) column equilibrated with 0.1 M NH_4HCO_3, pH 8, at flow rates of 0.25 to 1 ml/min.

2.4. *Automated sequence analysis*

Sequence analysis was performed with Applied Biosystems 470A gas-phase and 477A pulsed liquid-phase peptide sequenators. PTH-amino acids were analysed on-line with Applied Biosystems 120A analysers [14]. Data collection and analysis were done with an Applied Biosystems 900A module calibrated with 25 pmol PTH-amino acid standards.

2.5. *Amino acid analysis*

Peptides and protein B were analysed using a Beckman 121MB ion-exchange amino acid analyser with ninhydrin detection and an Applied Biosystems 420A derivatiser-analyser fitted with an on-line 130A PTC analyser [15]. Peptides were hydrolysed for 20–24 h at 110°C with 6 N HCl/2 mM phenol in the vapour phase under N_2. Samples were dried, dissolved in distilled water and lyophilised before analysis.

2.6. *Fast atom bombardment mass spectrometry*

Apo-protein B was analysed by FAB-MS using a VG ZAB-SE mass spectrometer. The sample was prepared in a thioglycerol matrix and bombarded with a caesium ion fast atom source.

3. RESULTS AND DISCUSSION

Protein B was homogeneous as determined by SDS-gel electrophoresis with a molecular mass of 12 kDa. Hydroxylamine treatment of protein B did not appear to cleave the protein at the Asn-Gly bond [16], residues 102–103. This would have confirmed the carboxy-terminus of the protein. However such a small fragment could have remained undetected on SDS-gel electrophoresis. Digestion of protein B with carboxypeptidase Y confirmed the C-terminal sequence -Glu-Leu-Lys. The cleavage at the tryptophan residues (positions 2 and 47) by cyanogen bromide in formic acid at 4°C under nitrogen was unexpected. This was observed in three separate digests to the extent of approx. 50% for both tryptophans. This proved useful in the case of tryptophan-47 to help establish overlaps. The key sequence information to establish the complete structure is summarised in fig.1.

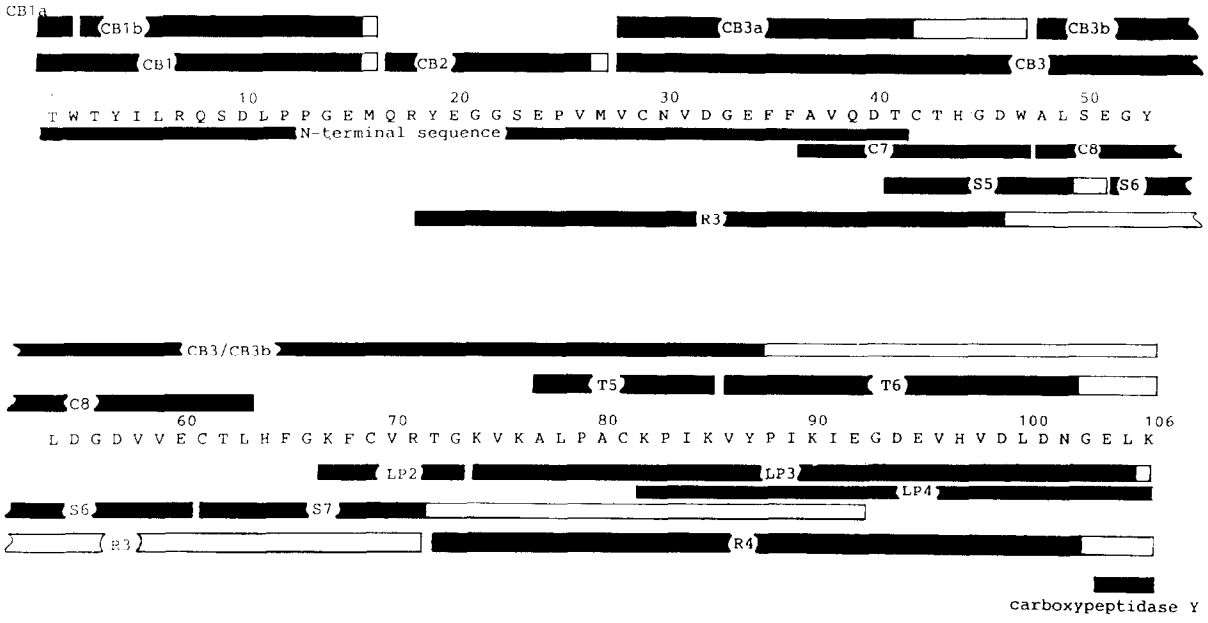

Fig.1. Deduction of amino acid sequence of *Pseudomonas putida* benzene dioxygenase protein B. Peptides are denoted by CB, cyanogen bromide cleavage; R, arginine-specific proteinase; LP, *A. mellea* lysine-specific proteinase; S, *S. aureus* V8 proteinase; C, chymotryptic peptides; T, tryptic peptides. Only the amino terminal sequence and sufficient key peptides to establish the sequence are shown. The bars denote position of these peptides. This is shown as a solid line to indicate the extent to which the fragment was sequenced.

Table 1

Amino acid analysis of protein B

	Analysis	Sequence
Asp	10.9	9
Asn		2
Ser	3.2	3
Thr	6.1	6
Glu	11.2	9
Gln		3
Pro	5.7	6
Gly	10.7	11
Ala	5.8	4
Met	2.0	2
Val	9.6	11
Ile	4.1	4
Leu	9.5	8
Tyr	3.3	4
Phe	4.1	4
His	2.8	3
Lys	7.8	7
Arg	3.0	3
Cys	4.7	5
Trp	–	2
Total		106

Ser and Thr were corrected for destruction by 10% and 5%, respectively. Val and Ile values were from 72 h hydrolysis. Cys (as cysteic acid) and Met (as methionine sulphone) were from performic acid oxidised samples, hydrolysed for 72 h

FAB-MS of apo-protein B indicated a molecular mass of 11860 ± 16 Da as compared with 12300 Da from gel filtration and ultracentrifugation sedimentation studies [5]. From sequence analysis, the molecular mass of 11809 Da for protein B is in fair agreement with the results of mass spectrometry. The amino acid analysis of protein B is given in table 1 and is in agreement with both the protein sequence and mass spectrometry data. The amino acid sequences of protein B from the benzene dioxygenase system of both strains of *Ps. putida* are shown in fig.2. In the present study this is a single polypeptide of 106 residues containing five cysteine residues, four of which are presumably involved in the [2Fe-2S] coordination. The protein structure deduced from the oligonucleotide sequence [8] of strain BE-81 is shown below the protein sequence from strain NCIB 12190. The two sequences differ at five positions. Four of these are highly conservative substitutions, i.e. Glu-Asp-51; Val-Ile-58; Tyr-Phe-87; Ile-Val-91. The more radical substitution Ser-Pro-23 should not alter the protein folding. The Robson-Garnier predicted secondary structure [17] in this region (-Gly-Gly-Pro/Ser-Glu-Pro-) has a high probability of being in a turn and a very low probability of being in an α-helix or β-sheet.

In this study we can identify the gene product P3, sequenced by Irie and co-workers [8], as the ferredoxin component of the benzene dioxygenase complex. The start of the open reading frame of the gene was given as the methionine, residue 16 in the present numbering system (fig.2). This shorter protein does not exclude any of the cysteine residues and would be presumably functionally viable. These authors detected four proteins from five possible open reading frames, using a DNA-directed in vitro translation system. The protein corresponding to their calculated molecular mass for this ferredoxin component (10030 Da) was not detected although an unidentified protein of slight-

```
    1                                   10                                  20
(a) Thr-Trp-Thr-Tyr-Ile-Leu-Arg-Gln-Ser-Asp-Leu-Pro-Pro-Gly-Glu-Met-Gln-Arg-Tyr-Glu-Gly-Gly-SER-Glu-Pro-Val-Met-
(b)                                                                 Met-Gln-Arg-Tyr-Glu-Gly-Gly-PRO-Glu-Pro-Val-Met-

            30                                  40                                  50
(a) Val-Cys-Asn-Val-Asp-Gly-Glu-Phe-Phe-Ala-Val-Gln-Asp-Thr-Cys-Thr-His-Gly-Asp-Trp-Ala-Leu-Ser-GLU-Gly-Tyr-Leu-
(b) Val-Cys-Asn-Val-Asp-Gly-Glu-Phe-Phe-Ala-Val-Gln-Asp-Thr-Cys-Thr-His-Gly-Asp-Trp-Ala-Leu-Ser-ASP-Gly-Tyr-Leu-

                    60                                  70                                  80
(a) Asp-Gly-Asp-VAL-Val-Glu-Cys-Thr-Leu-His-Phe-Gly-Lys-Phe-Cys-Val-Arg-Thr-Gly-Lys-Val-Lys-Ala-Leu-Pro-Ala-Cys-
(b) Asp-Gly-Asp-ILE-Val-Glu-Cys-Thr-Leu-His-Phe-Gly-Lys-Phe-Cys-Val-Arg-Thr-Gly-Lys-Val-Lys-Ala-Leu-Pro-Ala-Cys-

                            90                                  100                 106
(a) Lys-Pro-Ile-Lys-Val-TYR-Pro-Ile-Lys-ILE-Glu-Gly-Asp-Glu-Val-His-Val-Asp-Leu-Asp-Asn-Gly-Glu-Leu-Lys
(b) Lys-Pro-Ile-Lys-Val-PHE-Pro-Ile-Lys-VAL-Glu-Gly-Asp-Glu-Val-His-Val-Asp-Leu-Asp-Asn-Gly-Glu-Leu-Lys
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Fig.2. Complete amino acid sequence of protein B from *Pseudomonas putida*. The protein sequence from (a) strain NCIB 12190 (this study) is compared to that deduced from (b) the DNA sequence of strain BE-81 [8]. Differences are shown in upper case.

ly higher molecular mass is discernable in their SDS-polyacrylamide gel autoradiographs [8].

The complete amino acid sequence was compared with the sequences of 70 other ferredoxins listed in the Dayhoff and EMBL protein and DNA sequence databanks. No significant homology was detected for these 2Fe, 3Fe and 4Fe ferredoxins. The position of the cysteine residues is known to have an important role in defining the structure and redox potential [9]. The spacing between the cysteines in protein B would indicate that the tertiary structure of this ferredoxin would be very different from that of *Spirulina platensis* ferredoxin [18].

There are few noticeable similarities between the sequence of ferredoxin B and the other ferredoxin sequences, including putidaredoxin from *Ps. putida* [10]. However the occurrence of a histidine residue, in the sequence -Cys-Thr-His- may be significant. In adrenodoxin (E_m −270 mV) and putidaredoxin (E_m −240 mV) there is also a histidine, in the sequence -Thr-Cys-His-, whereas the lower-potential photosynthetic ferredoxins have none. The 'Rieske' iron-sulphur proteins from various species have invariant histidines in the vicinity of both cysteines [19], including the sequence -Cys-Thr-His- found in ferredoxin B. These are [2Fe-2S] proteins with considerably more positive midpoint potentials, +160–290 mV [7]. Histidine nitrogens are believed to be ligands to the cluster [20]. (Parenthetically, the protein A1 of the benzene dioxygenase is spectroscopically similar to the Rieske proteins.) Fig.3 compares the position of the cysteines and adjacent histidines in protein B with those in plant ferredoxin [9], putidaredoxin [10], the 2Fe-2S ferredoxin from *Ps. putida*, the 2Fe-2S ferredoxin from *C. pasteurianum* [11] and the Rieske protein from beef heart [19]. The presence of one histidine near the iron-sulphur cluster might cause the protein B to have an intermediate potential between the Rieske protein and the plant-type ferredoxins.

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