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The primary structure of *Pseudomonas* cytochrome *c* peroxidase

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The primary structure of *Pseudomonas* cytochrome *c* peroxidase is presented. The intact protein was fragmented with cyanogen bromide into five fragments; partial cleavage was observed at a Met-His bond of the protein. The primary structure was established partly by automatic Edman degradations, partly by manual sequencing of peptides obtained with trypsin, thermolysin, chymotrypsin, pepsin, subtilisin and *Staphylococcus aureus* V8 endopeptidase. The order of the cyanogen bromide fragments was further confirmed by overlapping peptides obtained by specific cleavage of the whole protein. *Pseudomonas* cytochrome *c* peroxidase consists of 302 amino acid residues giving a calculated M_r of 33690.

Cytochrome-*c* peroxidase; Primary structure; Heme-binding site; (*Pseudomonas aeruginosa*)

1. INTRODUCTION

Pseudomonas cytochrome *c* peroxidase (cytochrome *c*-551:H_2O_2 oxidoreductase, EC 1.11.1.5) has, unlike other peroxidases, two heme *c* moieties in a single polypeptide chain [1]. One of the hemes is low-potential having the function of a peroxidasic heme whereas the other one functions as a high-potential cytochrome *c* [2,3]. An interaction between the hemes is evident during the catalytic cycle of the enzyme [4,5]. The amino acid sequences of the two heme-binding sites of the enzyme have been determined [6]. Further, the enzyme has been cleaved into five fragments with cyanogen bromide, the internal order of which has been established [7]. In the present communication, the complete amino acid sequence of *Pseudomonas* cytochrome *c* peroxidase is presented. The determination of the amino acid sequence of the enzyme will be described elsewhere in detail.

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2. MATERIALS AND METHODS

Pseudomonas cytochrome *c* peroxidase was purified, cyanogen bromide fragments prepared and the heme moieties removed as described [7].

Enzymatic redigestions of the fragments were carried out with trypsin, chymotrypsin, thermolysin and subtilisin [7]. Digestion with V8 endopeptidase from *Staphylococcus aureus* (Boehringer, GFR) was as in [8] and specific cleavage at tryptophan residue as in [9]. The peptides were purified by gel filtration and reversed-phase HPLC and in some few cases with paper chromatography and paper electrophoresis. The peptides were analyzed for amino acid composition and purity as described [6]. Cyanogen bromide fragments were also hydrolyzed with 4 N methanesulfonic acid (Pierce) to determine the tryptophan content [10].

Sequencer degradations of the protein and of the larger peptides were carried out in a liquid-phase spinning-cup or a gas-phase sequencer with on-line phenylthiohydantoin (PTH)-amino acid analysis. The amino acid sequence of smaller peptides was determined by direct manual Edman degradation [11] and the analysis of the released PTH-amino acid derivatives was by reversed-phase HPLC [12]. Amide groups were assigned from peptide electrophoretic mobilities and by direct identification of phenylthiohydantoins. The carboxy-terminal amino acids were determined using carboxypeptidase A (Worthington) [13].

Cyanogen bromide fragments are numbered CB1 to CB5 according to their position in the sequence.

3. RESULTS

Automated sequence analysis of *Pseudomonas*

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cytochrome *c* peroxidase established the first 50 residues of the N-terminus (fig.1). The time-dependent release of C-terminal amino acids by treatment with carboxypeptidase A indicated a C-terminal sequence Ala-Glu for the peroxidase. Cleavage with cyanogen bromide resulted in five fragments, the amino acid compositions of which are listed in table 1. One overlapping fragment was obtained, derived from incomplete cleavage of the bond between fragments CB1 and CB2.

3.1. *CB1 (residues 1–95)*

The N-terminal sequence of the first 50 residues of this fragment was derived from data on the intact protein. Further information and necessary overlaps were obtained by sequencing peptides obtained with trypsin, chymotrypsin and thermolysin.

3.2. *CB2 (residues 96–132)*

The N-terminal sequence could be determined with the sequencer up to position 118. The total sequence of the fragment was provided by analysis of tryptic, chymotryptic and thermolytic peptides.

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          5                  10                  15                  20
Asp-Ala-Leu-His-Asp-Gln-Ala-Ser-Ala-Leu-Phe-Lys-Pro-Ile-Pro-Glu-Gln-Val-Thr-Glu-

          25                  30                  35                  40
Leu-Arg-Gly-Gln-Pro-Ile-Ser-Glu-Gln-Gln-Arg-Glu-Leu-Gly-Lys-Lys-Leu-Phe-Phe-Asp-

          45                  50                  55                  60
Pro-Arg-Leu-Ser-Arg-Ser-His-Val-Leu-Ser-Cys-Asn-Thr-Cys-His-Asn-Val-Asp/Gly-Thr-Asp/Gly-

          65                  70                  75                  80
Gly-Ala-Gly-Asn-Val-Pro-Thr-Ser-Val-His-Gly-Gly-Gln-Lys-Gly-Pro-Arg-Ala-Lys-Asp-

          85                  90                  95                  100
Leu-Gly-Glu-Gln-Ala-Lys-Gly-Pro-Ile-Gln-Asn-Ser-Val-Gln-Met-His-Ser-Thr-Pro-Gln-

          105                 110                 115                 120
Leu-Val-Glu-Gln-Thr-Leu-Gly-Ser-Ile/Val-Pro-Glu-Tyr-Val-Asp-Ala-Phe-Arg-Lys-Ala-Phe-

          125                 130                 135                 140
Ala/Pro-Lys-Ala-Gly-Lys-Pro-Val-Ser-Phe-Asp-Asn-Met-Ala-Leu-Ala-Ile-Glu-Ala-Tyr-Glu-

          145                 150                 155                 160
Ala-Thr-Leu-Val-Thr-Pro-Asp-Ser-Pro-Phe-Asp-Leu-Tyr-Leu-Ile/Lys-Gly-Asp-Asp-Lys-Ala-

          165                 170                 175                 180
Leu-Asp-Ala-Gln-Gln-Lys-Lys-Gly-Leu-Lys-Ala-Phe-Met-Asp-Ser-Gly-Cys-Ser-Ala-Cys-

          185                 190                 195                 200
His-Asn-Gly-Ile-Leu-Gly-Gly-Gln-Ala-Tyr-Phe-Pro-Phe-Gly-Leu-Val-Lys-Lys-Pro-Asp-

          205                 210                 215                 220
Ala-Ser-Val-Leu-Pro-Ser-Gly-Asp-Lys-Gly-Arg-Phe-Ala-Val-Thr-Lys-Thr-Gln-Ser-Asp-

          225                 230                 235                 240
Glu-Tyr-Val-Phe-Arg-Ala-Ala-Pro-Leu-Arg-Asn-Val-Ala-Leu-Thr-Ala-Pro-Tyr-Phe-His-

          245                 250                 255                 260
Ser-Gln-Gly-Val-Trp-Gln-Leu-Lys-Asp-Ala-Val-Ala-Ile-Met-Gly-Asn-Ala-Gln-Leu-Gly-

          265                 270                 275                 280
Lys-Gln-Leu-Ala-Pro-Asp-Asp-Val-Glu-Asn-Ile-Val-Ala-Phe-Leu-His-Ser-Leu-Ser-Gly-

          285                 290                 295                 300
Lys-Gln-Pro-Arg-Val-Glu-Tyr-Pro-Leu-Leu/Trp-Pro-Ala-Ser-Thr-Glu-Thr-Thr-Pro-Arg-Pro-

Ala-Glu
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Fig.1. Amino acid sequence proposed for *Pseudomonas* cytochrome *c* peroxidase.

Table 1

Amino acid composition of cyanogen bromide fragments of *Pseudomonas* cytochrome *c* peroxidase

	CB1[a]	CB2	CB3	CB4	CB5
Position in sequence	1–95	96–132	133–173	174–254	255–302
Asp	9.6 (9)	3.2 (3)	4.7 (5)	7.0 (7)	4.5 (4)
Thr	4.4 (4)	1.9 (2)	2.0 (2)	2.9 (3)	2.9 (3)
Ser	6.7 (7)	2.8 (3)	1.1 (1)	5.6 (6)	2.9 (3)
Glu	13.6 (14)	3.9 (4)	4.1 (4)	5.2 (5)	7.4 (7)
Pro	7.1 (7)	3.1 (3.5)	2.4 (2)	5.5 (5)	5.8 (6)
Gly	9.7 (10)	2.4 (2)	2.1 (2)	8.5 (8)	3.2 (3)
Ala	7.8 (6)	3.2 (2.5)	6.8 (7)	9.7 (10)	5.1 (5)
Val	6.1 (6)	3.4 (3.5)	1.2 (1)	7.0 (7)	2.5 (3)
Ile	2.6 (3)	0.7 (0.5)	1.1 (1.5)	1.9 (2)	0.7 (1)
Leu	8.0 (8)	2.4 (2)	5.7 (6)	5.6 (6)	5.8 (5.5)
Tyr	+ (–)	0.9 (1)	2.0 (2)	2.8 (3)	1.0 (1)
Phe	4.8 (3)	3.1 (3)	1.9 (2)	5.0 (5)	1.1 (1)
His	4.3 (4)	1.0 (1)	–	2.1 (2)	1.2 (1)
Lys	5.6 (6)	2.6 (3)	4.5 (4.5)	5.0 (5)	2.3 (2)
Arg	5.0 (5)	1.0 (1)	–	3.1 (3)	1.7 (2)
Met/HSe	0.97 (1)	0.9 (1)	1.0 (1)	1.0 (1)	–
Cys	1.6 (2)	–	–	1.9 (2)	–
Trp	–	–	–	1.0 (1)	0.4 (0.5)
Total	95	37	44	81	48

[a] Contains fragment CB1-CB2 as a contaminant

Residues per molecule by amino acid analysis and (in parentheses) from sequence are shown

3.3. *CB3 (residues 133–173)*

The N-terminal sequence of the first 24 residues was established in the sequencer. The remaining structure was determined by analysis of tryptic, chymotryptic and thermolytic peptides.

3.4. *CB4 (residues 174–254)*

The sequence of this heme *c* containing fragment has been reported previously [7].

3.5. *CB5 (residues 255–302)*

The N-terminal sequence of the carboxylterminal cyanogen bromide fragment was determined to position 292 by the sequencer. The sequence of CB5 was established by cleaving the fragment with trypsin, chymotrypsin, thermolysin, protease V8 and *o*-iodosobenzoate and by sequencing the isolated peptides.

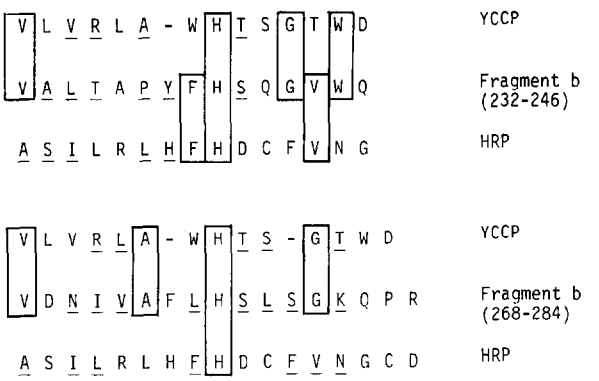

Fig.2. Comparison of the sequences of the two histidine regions of fragment *b* with the sequences of the distal histidine regions of yeast cytochrome *c* peroxidase and horseradish peroxidase. Fragment *b* was obtained by *Pseudomonas* elastase cleaving of *Pseudomonas* cytochrome *c* peroxidase, PaCCP. YCCP, yeast cytochrome *c* peroxidase; HRP, horseradish peroxidase. Identical sequences are boxed. Underlined residues are those with only one base difference per codon.

4. DISCUSSION

The determination of the primary structure of *Pseudomonas* cytochrome *c* peroxidase is primarily based on the analysis and alignment of five cyanogen bromide fragments.

The N-terminal sequence of CB2 was first deduced to be Ser-Thr- [7]. Further studies on a sequencer, however, revealed the sequence His-Ser-Thr- (fig.1). These contrasting results reflect the abnormal behaviour of histidyl-peptides encountered in the manual Edman degradation procedure [14].

Although the enzyme preparation was homogeneous, microheterogeneities were observed at positions 58, 60, 109, 121, 155 and 290. All these exchanges are compatible with a one-base difference in corresponding codons.

The calculated molecular mass of the enzyme is found to be smaller than that obtained by SDS-acrylamide gel electrophoresis [1]. It is known that a close relationship exists between the electrophoretic migrations of proteins in SDS-acrylamide gels and their molecular mass only when the molecules have the same hydrodynamic shape and charge-to-mass ratio [15]. It seems that the two covalently bound heme *c* groups in the peroxidase decrease its electrophoretic mobility resulting in too large a molecular mass when normal 'unhindered' proteins are used as molecular mass standards.

The peroxidatic low-potential heme is bound to the peptide chain at Cys-51 and Cys-54, His-55 being the proximal histidine residue [6]. The low-potential heme is pentacoordinated at room temperature while a histidine residue occupies the sixth coordination position at low temperatures. *Pseudomonas* elastase cleaves the polypeptide chain of the peroxidase at Ser-202 (into fragment *a* and fragment *b*) causing loss of the peroxidasic activity [7]. Hence, the distal histidine is concluded to be one of the two histidine residues in fragment *b* (203–302). Fig.2 shows the primary structure around these two histidine residues compared with the neighbourhood of the distal histidine residues in horseradish peroxidase [16] and yeast cytochrome *c* peroxidase [17]. From this comparison, His-240 is assumed to be the sixth ligand of the low-potential heme.

The cytochrome-like high-potential heme is bound to the polypeptide chain at Cys-177 and Cys-180. His-181 is assumed to be the proximal histidine residue [7] and Met-254 the distal ligand of the heme-iron [7]. His-240, the distal histidine of the low-potential heme, matches His-47 of *P. aeruginosa* and *P. aureus* cytochromes *c*-551 which both are hydrogen-bonded to the inner propionic acid [18]. This might be the structural basis of the heme–heme interaction observed in the enzyme molecule [4,5].

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