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Proteolysis of *Paracoccus denitrificans* cytochrome oxidase by trypsin and chymotrypsin

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Paracoccus oxidase containing only two subunits was subjected to proteolysis by trypsin and chymotrypsin. Both subunits in the purified enzyme were cleaved at only a few sites and enzymatic activity was not inhibited. The cleavage sites were identified by protein sequencing. Subunit I was cleaved near the amino-terminus and subunit II in the loop connecting the two predicted trans-membrane helices. In native membrane fragments, but not in intact spheroplasts, this loop was accessible to both proteases. These results provide experimental evidence for the folding of subunit II in the membrane.

Cytochrome aa_3; Protease; Protein folding

1. INTRODUCTION

Paracoccus cytochrome oxidase (EC 1.9.3.1) can be purified to contain only two subunits [1]. This is in contrast to the mammalian enzyme which appears to contain 13 different subunits [2]. The smaller and simpler bacterial enzyme is, however, fully competent in enzymatic activity and very similar to the mitochondrial one in spectroscopic properties (for review see [3]). The genes encoding the *Paracoccus* oxidase were recently sequenced and shown to be highly homologous to the corresponding mammalian oxidase subunits [4,5]. These findings make the *Paracoccus* enzyme an attractive subject for studies on structure-function relationships in cytochrome oxidase.

Subunit II contains in its primary structure two highly conserved segments. These are a stretch of aromatic residues and a putative copper-binding site [5]. Both segments are predicted to be located outside the membrane [5,6], and thus they may be accessible to water-soluble proteases.

In this work proteolysis of the two-subunit oxidase by trypsin and chymotrypsin is reported. The results suggest that all protein segments essential for activity are protected against these proteases. The identification of cleavage sites provides evidence for the folding of subunit II in the detergent solubilized as well as in the membranous enzyme.

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2. MATERIALS AND METHODS

Trypsin (type XIII) and α-chymotrypsin (type VII) were purchased from Sigma.

Paracoccus S1657 was grown aerobically as previously described [7]. A two-subunit cytochrome oxidase was either purified as described elsewhere [8], or was a generous gift from Professor B. Ludwig, Lübeck, FRG.

Purified enzyme was diluted to 10 μM cytochrome oxidase (aa_3) in 1% cholate (w/v), 50 mM Tris-Cl, 50 mM NaCl and 0.1 mM EDTA, pH 7.4. Incubations with trypsin or chymotrypsin (final concentration 0.2 mg/ml, diluted from fresh aqueous solutions) were performed for 1 h at 22°C unless otherwise stated. The digestion was stopped by adding phenylmethylsulfonyl fluoride (PMSF) to 0.5 mM and applying the samples to small (2×0.5 cm) DEAE-cellulose columns (DE52, Whatman) for removal of the proteases. The columns were preequilibrated with 1% cholate, 20 mM tris-Cl, pH 7.4, and this solution was also used for washing the bound oxidase which was then eluted by 0.5 M NaCl in the above medium.

Dioxygen-reduction activity was assayed polarographically as in [9] but in the presence of sonicated soybean phospholipids

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(final concentration 1 mg/ml). The enzyme was incubated before the assay for 30 min with more concentrated phospholipids (20 mg/ml), to allow exchange of bound cholate.

Spheroplasts were prepared essentially according to [10] but from fresh, unfrozen cells to minimize breakage of the plasma membrane. The spheroplasts were suspended again in the high-sucrose medium [10] to about 25 mg protein per ml (biuret) and divided into five portions of about 10 ml each. Two portions were mixed with either trypsin or chymotrypsin from fresh solutions to a final protease concentration of 1 mg/ml and stirred at room temperature for 90 min. The digestions were stopped by the addition of 3 vols of cold medium followed by PMSF to 0.5 mM and transferred to ice. The spheroplasts were collected by centrifugation (10 000 rpm, 10 min, SS34 rotor) and then broken by osmotic shock in the presence of 0.1 mM PMSF (see below).

The other three portions of spheroplasts were suspended in 300 ml of cold lysis buffer [10] and homogenized using an Ultraturrax tissue homogenizer, at 0°C for 5 min. Membranes were collected by centrifugation (20 000 rpm, 10 min, SS34), and suspended in the high-sucrose medium to an original volume of 10 ml for proteolytic digestion. Protease treatments were done as in two of the portions described above, and the third one was incubated similarly but without any protease for control. Finally the membranes from all the treatments were collected, suspended in the lysis medium and washed once more in the presence of 0.1 mM PMSF.

Before SDS-PAGE the membranes were extracted with 10% cholate and the extracts were applied to small DEAE-cellulose columns as described above. This step was included to remove membranous material that interferes with the migration of protein bands in the gel, and to remove residual proteases left in the membrane samples. The latter was essential, since the rabbit serum reacted with both proteases in a Western blot (not shown).

SDS-PAGE and Western blotting were done as described in [11]. Intact and digested polypeptides were identified using specific antiserum against subunit II (a generous gift from Professor B. Ludwig, Lübeck, FRG).

Protein sequencing was performed as described before [7].

3. RESULTS

Digestion of purified *Paracoccus* oxidase by trypsin or chymotrypsin resulted in limited cleavage of both subunits. Small fragments were removed from subunit I and a larger one from subunit II. The latter was almost identical for both proteases (fig.1). The digestions were complete after about 30 min at 22°C. Longer incubations (up to 4 h; not shown) or incubation at 37°C for 1 h, even in the presence of both proteases, did not yield further cleavage of the enzyme (fig.1). Under all these conditions there was no inhibition of dioxygen reduction activity by the protease treatment in comparison to enzyme incubated similarly but in the absence of protease (not shown).

Short incubations with chymotrypsin indicated

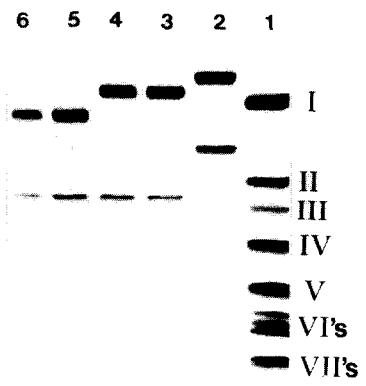

Fig.1. SDS-PAGE analysis of *Paracoccus* oxidase after proteolysis. Lanes: 1, bovine heart oxidase given as a marker; 2, control, incubation without protease; 3 and 4, trypsin cleavage; 5 and 6, chymotrypsin cleavage; 3 and 5, incubation with the proteases at 22°C; 4 and 6, incubation with proteases at 37°C. Other technical details are described in section 2.

that the proteolysis of subunit I occurred sequentially. Subunit II on the other hand appears to be cleaved only at a single site by each protease since no cleavage products larger than the final one were observed.

The digested enzyme was subjected to automated protein sequencing. Knowing the primary structure of both polypeptides [4] and the specificity of the proteases used, it was easy to resolve the two amino acids obtained at each cycle of the Edman degradation into N-termini sequences of the cleavage products. The results are presented in table 1 so that they indicate the peptide bond of the amino acid residue which is cleaved on the carboxyl-side by each protease. The numbers of the residues are given according to the gene, counting Met as no.1 [4]. Trypsin had an additional cleavage site in subunit I at Arg_{18}, but it was minor in comparison to Arg_{13}. An identical cleavage site in subunit II, but not in I, was recently reported by another lab [12].

The identification of the cleavage sites in subunit II (table 1), explains the observed identical migration of its fragment in SDS-PAGE after treatments by both proteases (fig.1). This suggests that subunit II is not cleaved at the C-terminus.

Protease cleavage sites near the predicted N-terminus of subunit I (table 1 and [4]), strongly suggest that this subunit does not contain a leader

Table 1

The cleavage sites of trypsin and chymotrypsin in *Paracoccus* cytochrome oxidase

	Subunit I	Subunit II
Trypsin	Arg_{98}	Arg_{13}
Chymotrypsin	Phe_{99}	Trp_{19}

The amino acid residues in the table are those the peptide bond of which is cleaved by the proteases on the carboxyl side. They were identified by protein sequencing and the published sequences of the genes.

sequence in its gene. Subunit II, on the other hand, was reported to be translated containing a leader sequence which is later removed from the mature protein [5].

The hydropathy plot of the *Paracoccus* oxidase subunit II [4,5], as well as of all the homologous subunits sequenced this far, predict two trans-membrane segments [6]. Both trypsin and chymotrypsin cleaved subunit II in the loop hat is predicted to be between the two trans-membrane helices [5]. The folding model of the subunit places this loop on the cytoplasmic side of the membrane, with both N- and C-termini on the periplasmic side [5,6]. Determination of the accessibility of this loop to proteolysis in bacterial cells devoid of the outer membrane, but with an intact plasma membrane (spheroplasts), and in purified membrane fragments, would thus be a good way to test the folding model. The results of such an experiment are shown in fig.2. It is clear that the cleavage site is inaccessible in spheroplasts. In contrast, with purified membranes the cleavage took place, and the product was the same as with the isolated enzyme in a detergent solution (fig.2). Proteolysis efficiency was much reduced, however, with the membranous enzyme. This could be due to the higher concentration of protein (other than the oxidase) in the native membrane samples or the presence of vesicles in the membrane samples.

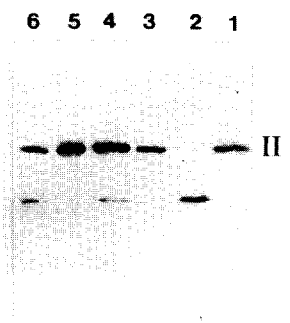

Fig.2. Accessibility of the cleavage site in subunit II in the native membrane. Spheroplasts (lanes 3,5) and broken membranes (lanes 4,6) were prepared as described in section 2. Proteolysis was with trypsin (lanes 3 and 4) or chymotrypsin (lanes 5 and 6). Lane 1 shows control membranes and lane 2 purified enzyme cleaved with trypsin (see fig.1). The samples were analyzed by SDS-PAGE followed by Western blotting and identification of subunit II by specific antibodies. See section 2 for details.

The results (fig.2) strongly suggest that the loop connecting the two helices is exposed to the cytoplasmic side (inside) of the membrane, which supports its location in the folding models [6,13].

4. DISCUSSION

The results described in this work suggest that a large segment of the *Paracoccus* oxidase subunit II is not essential for electron transfer from cytochrome *c* to dioxygen. This segment extends from the N-terminus to Phe_{99} (at least), and it includes one of the two trans-membrane helices. It is interesting that none of the highly conserved residues of cytochrome oxidase subunit II is located in this segment. The first such residue starting from the N-terminus is the glutamic (or aspartic) acid at the beginning of the second helix, and is shown in fig.3 (see [6] for the location of the strictly conserved residues). Yet, the hydropathy plot which predicts two transmembrane helices is highly conserved [14].

Removal of the first helix without inhibition of activity excludes binding of haem *a* between the two helices of subunit II as suggested before [15]. Previous indications against such binding of the haem came from the finding that the histidine residue at the beginning of the first helix, which was earlier thought to be highly conserved, was not present in some parasite oxidases [14]. However, recent observations that RNA editing commonly occurs in *Trypanosoma* [16], suggest that such conclusion about the conservation of the histidine cannot be based on DNA sequence alone. The results presented here are thus the first experimental evidence against haem *a* binding to subunit II, even

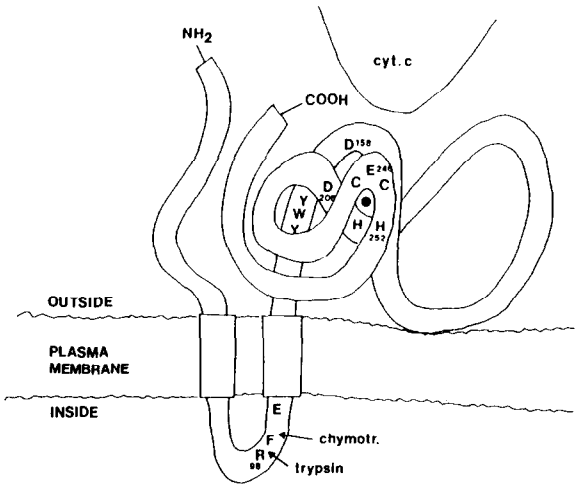

Fig.3. A schematic model for the folding of subunit II of *Paracoccus* cytochrome oxidase. The cleavage sites of the proteases are shown at the bottom. The closed circle in the middle represents a possible copper ion. See text for discussion.

if this histidine finally turns out to be highly conserved.

Subunit II contains a highly conserved segment of aromatic amino acid residues [6]. One aim of this study was to possibly gain insight into the role of this segment by its cleavage with chymotrypsin. However, the results suggest that this segment, as well as the putative copper-binding site in subunit II, are very well protected or shielded against chymotrypsin and trypsin proteolysis. These observations prompt us to slightly modify previous structural models for subunit II [6,13]. The model shown in fig.3 emphasizes protection of the conserved segments against proteolysis, and suggests a possible arrangement of the cytochrome *c*-binding site.

The model in fig.3 differs from the model of Holm et al. [6] in several ways. The aspartic acid residue which is located very near to the aromatic residues segment (D_{158}; fig.3), participates in cytochrome *c* binding, as suggested by a chemical modification study of the bovine enzyme [13]. This residue is not strictly conserved so it was not shown in [6]. However, a survey of all the available sequences of subunit II shows that all of them contain an aspartic or glutamic acid residue in their sequence very near to the aromatic residues segment. Placing this acidic residue near the glutamic acid in the middle of the copper-binding site (E_{246} here), which was also suggested to take part in cytochrome *c* binding [13], brings the aromatic residue segment in the vicinity of the putative metal site. This arrangement may be important for the enzymatic activity as previously discussed [17].

The model in fig.3 shows only subunit II but it should be kept in mind that the protection against proteolysis described here may also be provided, at least in part, by subunit I.

The orientation of subunit II with respect to the membrane, such that the loop between the predicted helices is exposed to the inside of the bacterial cell (fig.2), supports previous folding models of this subunit [6,13]. Localization of this loop in the otherwise better studied bovine enzyme, was not possible by the same method because it lacks suitable sites for the proteases [17].

The observation that subunit I does not contain a leader sequence, based on identification of the tryptic cleavage, suggests that the N-terminus of subunit I is on the cytoplasmic (inside) side of the membrane [6].

In summary the results of this work suggest a compact and well protected arrangement of the cytochrome oxidase regions which are predicted to be exposed on the periplasmic side of the *Paracoccus* plasma membrane.

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