Expression of the *Escherichia coli cyo* operon in *Paracoccus denitrificans* results in a fully active quinol oxidase of unexpected heme composition

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Abstract The *cyo* operon coding for the membrane-bound bo_3-type quinol oxidase of *Escherichia coli* has been expressed in a *Paracoccus denitrificans* strain deleted in its endogenous ba_3 quinol oxidase. Using the *P. denitrificans qox* promoter, the His tagged protein complex is synthesized to a level comparable to that in *E. coli* and the enzyme purified in a single step on a metal-chelating column. Whereas the activity of the isolated complex matches that of the oxidase purified directly from *E. coli*, the heterologously expressed oxidase does not show the characteristic heme composition but now carries heme *a* in its binuclear site.

Key words: Heme-copper terminal oxidase; Quinol oxidase; Cytochrome *ba*; Heme promiscuity; Heterologous expression

1. Introduction

Cytochrome bo_3 is one of two main terminal oxidases in the respiratory chain of *Escherichia coli*; it consists of four subunits encoded by the *cyo*ABCDE operon [1,2]. Subunits II, I, III and IV are gene products of the first four open reading frames. The *cyo*E gene encodes a protoheme IX farnesyl transferase (heme *o* synthase) essential for catalytic competence of this oxidase [3–5]. The cytochrome bo_3 complex belongs to the superfamily of heme-copper terminal oxidases ([6], and literature cited therein) and functions as a redox-coupled proton pump. Under normal growth conditions it contains a low-spin heme *b* and a binuclear oxygen-reducing site with Cu_B and a high-spin heme *o*, all located in subunit I [7].

The quinol oxidase of the soil bacterium *Paracoccus denitrificans* shares considerable sequence identity [8] with the bo_3 enzyme from *E. coli*, but its high-spin heme site is occupied by heme *a*. Hemes *a* and *o* are both derivatives of protoheme IX, with the vinyl group at pyrrole ring A replaced by the hydroxyethylfarnesyl moiety. In addition, heme *a* biosynthesis requires the oxidation of the methyl group at pyrrole ring D to yield a formyl group, a reaction not observed in *E. coli*.

In this study we address the question of heme composition of the *E. coli* oxidase when expressed in a *P. denitrificans* host, and show that the purified enzyme exhibits a ba_3 heme composition. In contrast to the previously described non-functional quinol oxidase species with a *b*-type heme in the binuclear center [3,4,9,10] this enzyme retains its electron transfer competence at wild-type level.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Paracoccus denitrificans strain ORI2K31 (see below) was grown at 32°C with succinate as the major carbon source [11] in the presence of 40 mM nitrate [10] and 25 μg/ml streptomycin, while *Escherichia coli* strain GO105 carrying pJRHisA [12] was grown at 37°C on yeast extract/tryptone medium including 50 μg/ml ampicillin.

2.2. Construction of the P. denitrificans strain ORI2K31 expressing the cyo operon

For efficient transcription in the heterologous system, the four structural genes coding for subunits I–IV of the *E. coli cyo* operon were placed under control of the *P. denitrificans qox* promoter [8,10] in a construction comprising several steps. Starting point was an engineered *qox* operon with the gene for *qox*A being replaced by *cyo*A coding for the equivalent subunit of the *E. coli* quinol oxidase [13]. The signal peptide of QoxA replaces that of CyoA, and the amino acid sequence of the mature subunit now starts with CKAEVLV instead of CNSALLD, then merging into the native CyoA sequence. This construct was cloned into the broad host range plasmid pRI [14], restricted with *Sma*I, and ligated to the *Sma*I/*Eco*RV fragment of pJRHisA [12]. The resulting plasmid, pOR660, now leads to expression of the four structural *cyo* genes; a His tag at the C-terminus of CyoA allows efficient purification of the complex by immobilized metal affinity chromatography [12]. ORI2K31 was obtained after conjugation [15] of pOR660 into *Paracoccus* strain ORI2/4 containing a chromosomal deletion of the *qox* operon, therefore lacking any endogenous quinol oxidase [10].

2.3. Preparation of solubilized membranes

Disruption of cells and isolation of membranes, protein determination and SDS-PAGE were done as described earlier [15,16]. Solubilization of membranes was performed at a protein concentration of 7 mg/ml in a buffer containing 50 mM KP_i, pH 8.0, 50 mM NaCl and 100 μM Pefabloc SC (Biomol, Germany) by adding *n*-dodecyl-β-D-maltoside (DM; Calbiochem, USA) to a ratio of 1.5 mg/1 mg membrane protein. The suspension was stirred at 4°C for 20 min and centrifuged at $186\,000 \times g$ for 1 h at 4°C.

2.4. Protein isolation

The supernatant of solubilized membranes was applied to a copper chelated resin (Chelating Sepharose Fast Flow, Pharmacia, Sweden) equilibrated in 50 mM KP_i, pH 8.0, 50 mM NaCl and 2 g/l DM. Washing was done with 2 volumes of 50 mM KP_i, 50 mM NaCl, 0.3 g/l DM and 60 mM imidazole, pH 8.0. The oxidase was eluted with the same buffer supplemented with 150 mM imidazole. Fractions were pooled according to their enzymatic activity and concentrated by ultrafiltration with a cutoff of 3×10^4 Da (Centriprep, Amicon, USA).

2.5. Quinol oxidase assay

Quinol oxidase activity was assayed spectrophotometrically with a Hitachi U-3000 spectrophotometer at 275 nm. Measurements were done at room temperature in 1 ml buffer containing 50 mM KP_i, pH 7.5, 1 mM EDTA, 0.3 g/l DM and 50 μM decyl ubihydroquinone (Sigma) as substrate, using an extinction coefficient of $\Delta\varepsilon_{275} = 12.5$ $mM^{-1} \cdot cm^{-1}$ [17].

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Abbreviations: HPLC, high performance liquid chromatography; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; DM, *n*-dodecyl-β-D-maltoside

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2.6. Optical spectra

Quantification of the purified quinol oxidase was based on the dithionite-reduced minus air-oxidized pyridine hemochrome spectrum, using extinction coefficients $\Delta\varepsilon_{587.5-620} = 25.02\ mM^{-1}\cdot cm^{-1}$ for heme *a* and $\Delta\varepsilon_{556.4-540} = 23.98\ mM^{-1}\cdot cm^{-1}$ for heme *b* [18]. The nature of the high-spin heme was determined from CO difference spectra as described [10]. An extinction coefficient of 5.9 $mM^{-1}\cdot cm^{-1}$ at 607 nm was calculated for heme a_3 on the basis of a native redox spectrum of purified *Paracoccus* ba_3 quinol oxidase, for which a 1:1 stoichiometry of heme *b* to heme *a* has been established [10].

2.7. HPLC heme analysis

Isolated enzyme complexes were treated twice with 4 volumes of 90% acetone, 10% water, 10 mM NH_4OH, and hemes were extracted first into acetone/HCl/H_2O (90:2:8, v/v) and then into diethyl ether as described [19]. Evaporated extracts were redissolved in ethanol/methylene chloride (50:50, v/v) and applied to a DEAE-Sepharose CL-6B column [20] equilibrated with the same solvent. The column was washed with 10 volumes of 87% aqueous ethanol and hemes were eluted with ethanol/acetic acid/H_2O (70:17:13, v/v) [21]. HPLC analysis was done in ethanol/acetic acid/H_2O (70:17:13, v/v) using a C_{18} reverse phase column (Macherey Nagel ET 250/4 Nucleosil 120-5C_{18}). Hemes were detected at 402 nm at a flow rate of 0.2 ml/min.

3. Results

Since there is no report of *Escherichia coli* promoters being functional in *Paracoccus denitrificans*, we chose to express the *E. coli cyo* genes in *P. denitrificans* under control of the *qox* promoter [8,10]. The QoxA signal sequence was placed in front of the four authentic *cyo* structural genes; in addition a His tag at the C-terminus of this subunit allows for an efficient purification [12]. This construct yields expression levels which are directly comparable to that of the *qox* operon expressed under the same conditions. As has been shown for *E. coli* [12], the tagged quinol oxidase can be purified to homogeneity from *P. denitrificans* membranes in a single step. Electrophoretic analysis confirms that the subunit molecular masses are identical irrespective of their expression in either *P. denitrificans* or *E. coli* (but clearly distinguishable from the Qox oxidase, data not shown).

The native redox spectrum (Fig. 1) of the Cyo oxidase isolated from *Paracoccus* reveals two major heme species: apart from a broad peak at 564 nm indicative of *b*- and/or *o*-type heme, a peak of lower intensity at 607 nm suggests the presence of heme *a*. A pyridine hemochrome spectrum (not shown) clearly identifies heme *a* (now with a peak at 589 nm) and its molar ratio of close to unity relative to the *b*/*o* peak observed at 556 nm. The apparent underestimation of heme *a* in the native spectrum is explainable by the low absorptivity (see Section 2) of fivefold coordinated high-spin heme relative to sixfold coordinated low-spin heme.

To unequivocally confirm the nature of the heme components giving rise to the peak at 564 nm in the native redox spectrum (see Fig. 1), the Cyo oxidase isolated from *P. denitrificans* was subjected to heme extraction followed by HPLC separation; heme peaks were identified by comparison of their retention times to those of selected cytochrome complexes (aa_3, bc_1, bo_3; not shown). Two dominant peaks are observed in the extract of the Cyo quinol oxidase purified from *P. denitrificans* that represent hemes *b* and *a*, while only a minor peak is assigned to heme *o*. This may nevertheless explain the molar excess of the heme components contributing to the peak at 556 nm in comparison to the peak at 589 nm observed in the pyridine hemochrome spectrum (see Table 1).

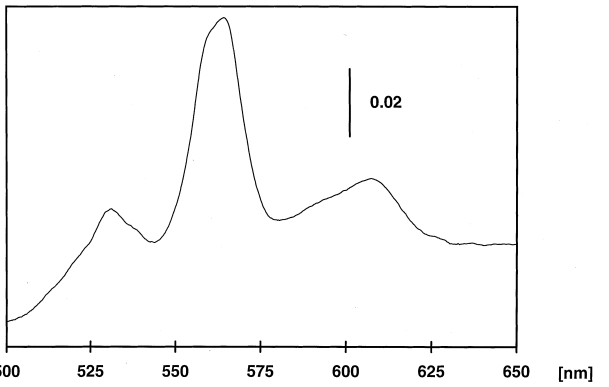

Fig. 1. Native redox spectrum of Cyo quinol oxidase isolated from *P. denitrificans* strain ORI2K31. The dithionite reduced minus oxidized difference spectrum was recorded at a protein concentration of 0.18 mg/ml.

The oxygen-reactive heme in the purified Cyo oxidase expressed in *P. denitrificans* was further analyzed by recording CO difference spectra (Fig. 2), comparing them to the authentic enzymes, the *E. coli* bo_3 and the *P. denitrificans* ba_3 quinol oxidase. The spectrum of the preparation from strain ORI2K31 (Fig. 2B) shows a peak and through at 589 and 609 nm in the α-region and at 426 and 441 nm, respectively, in the Soret region. These features are typical for CO-reactive heme *a* in the high-spin site and are identical to the *P. denitrificans* ba_3 quinol oxidase (Fig. 2A, and [10]). A small shoulder in the ORI2K31 spectrum at 416 nm and spectroscopic features in the α-region may correspond to a minor fraction of CO-reactive heme *o* in the high-spin site of this enzyme. This is in accordance to the observed small heme *o* peak in the heme analysis (see above) indicating that small amounts of the protein may be expressed as a bo_3-type quinol oxidase. In contrast, the quinol oxidase purified from *E. coli* GO105 (Fig. 2C) only shows typical features of CO-reactive heme *o* with peak and trough at 416 and 427 nm in the Soret region and the characteristic spectroscopic pattern in the α-region [22].

Despite the altered heme composition, the activity of the Cyo oxidase purified from either bacterium does not differ significantly (Table 1), indicating that the enzyme complex was assembled correctly and that the binuclear site tolerates

Table 1
Heme composition and activity of purified quinol oxidases expressed in different bacteria

Organism	Operon expressed	Heme composition[a]	Heme *b*:*a* ratio	Activity[b]
E. coli GO105	*cyo*	bo_3	–	549
P. denitrificans ORI2K31	*cyo*	ba_3	1:0.86	592
P. denitrificans G440	*qox*	ba_3[c]	1:1	180

[a]See also Fig. 1.
[b]Turnover number (1/s) based on quinol oxidation.
[c]ba_3 quinol oxidase purified using F_v fragments (Ostermann, T., unpublished).

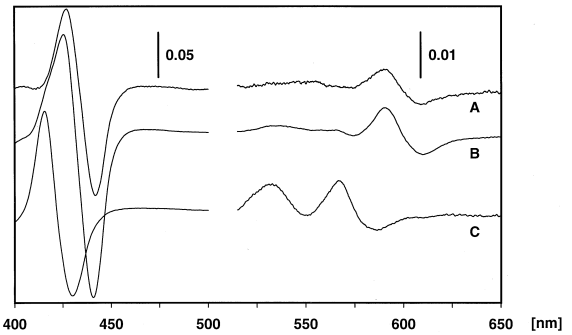

Fig. 2. CO difference spectra of purified quinol oxidases. A: *P. denitrificans* ba_3 quinol oxidase purified using F_v fragments (Ostermann, T., unpublished); B: Cyo oxidase expressed in *P. denitrificans* ORI2K31; C: *E. coli* bo_3 quinol oxidase (strain GO105). Vertical bars indicate absorption scale for the Soret and α-region. Spectra were normalized to identical absorption in the α-region.

either heme species at least without effect on the electron transfer capacity.

4. Discussion

The *Escherichia coli* cytochrome bo_3 complex and both the *Paracoccus denitrificans* aa_3 cytochrome *c* oxidase and ba_3 quinol oxidase belong to the superfamily of heme-copper terminal oxidases [6]. The highest degree of sequence identity is seen in their subunits I, which carry a low-spin heme and the binuclear center consisting of a high-spin heme and a copper ion.

Our experiments show that the *E. coli* Cyo quinol oxidase can be expressed in *P. denitrificans* under control of the *Paracoccus qox* promoter to the same amount and activity as the wild-type *E. coli* bo_3 quinol oxidase (Table 1). However, we could demonstrate that the expression of the corresponding genes in *P. denitrificans* leads to an enzyme complex with an altered heme composition in its high-spin site. The presence of heme *a* is already revealed by the native redox spectrum (Fig. 1) which resembles that of the *Paracoccus* ba_3 quinol oxidase [23]. HPLC heme analysis, pyridine redox and CO difference spectra all confirm that heme *o* typically found in the high-spin site of the bo_3 enzyme is indeed replaced by heme *a* when the enzyme is expressed in *P. denitrificans*. The pyridine redox spectrum of this oxidase shows a heme *b* to *a* ratio of near unity (Table 1), with only a small fraction of heme *o*. This is corrobarated by the CO difference spectrum demonstrating that the CO complex of heme a_3 absorbing at 426 nm is the dominant heme species in the high-spin site (Fig. 2B). The small blue-shift of this peak in comparison to the *Paracoccus* ba_3 quinol oxidase peak may be due to a different protein environment. A small shoulder at 416 nm and weak spectral features in the α-region similar to the *E. coli* bo_3 quinol oxidase are indicative of minor amounts of heme *o*. Presently, there is no evidence that *P. denitrificans* incorporates heme *o* into the high-spin sites of any of its endogenous terminal oxidases under normal growth conditions [24].

With the exception of cbb_3-type cytochrome *c* oxidases, all available evidence indicates that heme *b* is not able to sustain enzymatic activity of heme-copper oxidases when incorporated into the high-spin site. This has been studied in detail for the quinol oxidases of *E. coli* [3,4,9] and *P. denitrificans* [10]. The close to wild-type activity observed for the Cyo oxidase purified from *P. denitrificans* therefore excludes the presence of heme *b* as a constituent of the high-spin site of this particular enzyme complex. Therefore, a farnesylated derivative of heme *b* incorporated into the binuclear center seems to be a prerequisite for a functional heme-copper oxidase while it is of less importance whether heme *o* or heme *a* (with a formyl group in place of the methyl group at pyrrole ring D) fulfills this requirement. This mutual replacement of heme *a* by heme *o* in the high-spin site (or vice versa) has already been demonstrated for *Acetobacter acetii* [25] and cyanobacteria [26]. For example, under different growth conditions two different quinol oxidases with respect to their heme content were observed for *Acetobacter*.

In this report we show that the heme composition of the *E. coli* Cyo quinol oxidase is dependent on the bacterial host that expresses the corresponding enzyme complex. While heme *o* is the only farnesylated heme available in *E. coli*, *P. denitrificans* unexpectedly incorporates almost exclusively heme *a* into the high-spin site of this quinol oxidase giving rise to a ba_3 heme composition. As heme *a* supposedly is synthesized via heme *o* [27], this preference may indicate that heme *o* is not available for a heme-incorporating enzyme because of its faster conversion to heme *a*. As an alternative explanation, such a hypothetical enzyme may show a marked specificity both for the farnesylation and the formyl group on the tetrapyrrole ring.

Our data are therefore in accordance with the widely accepted notion of heme promiscuity in heme-copper terminal oxidases, extended here to the high-spin site of the *E. coli* quinol oxidase, an enzyme complex destined to contain heme *o* due to the lack of heme *a* biosynthesis in this organism.

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