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ORIGINAL ARTICLE

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Ca^{2+} and the bacterial peroxidases: the cytochrome *c* peroxidase from *Pseudomonas stutzeri*

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Abstract The production of cytochrome *c* peroxidase (CCP) from *Pseudomonas* (*Ps*.) *stutzeri* (ATCC 11607) was optimized by adjusting the composition of the growth medium and aeration of the culture. The protein was isolated and characterized biochemically and spectroscopically in the oxidized and mixed valence forms. The activity of *Ps. stutzeri* CCP was studied using two different ferrocytochromes as electron donors: *Ps. stutzeri* cytochrome c_{551} (the physiological electron donor) and horse heart cytochrome *c*. These electron donors interact differently with *Ps. stutzeri* CCP, exhibiting different ionic strength dependence. The CCP from *Paracoccus* (*Pa*.) *denitrificans* was proposed to have two different Ca^{2+} binding sites: one usually occupied (site I) and the other either empty or partially occupied in the oxidized enzyme (site II). The *Ps. stutzeri* enzyme was purified in a form with tightly bound Ca^{2+}. The affinity for Ca^{2+} in the mixed valence enzyme is so high that Ca^{2+} returns to it from the EGTA which was added to empty the site in the oxidized enzyme. Molecular mass determination by ultracentrifugation and behavior on gel filtration chromatography have revealed that this CCP is isolated as an active dimer, in contrast to the *Pa. denitrificans* CCP which requires added Ca^{2+} for formation of the dimer and also for activation of the enzyme. This is consistent with the proposal that Ca^{2+} in the bacterial peroxidases influences the monomer/dimer equilibrium and the transition to the active form of the enzyme. Additional Ca^{2+} does affect both the kinetics of oxidation of horse heart cytochrome *c* (but not cytochrome c_{551}) and higher aggregation states of the enzyme. This suggests the presence of a superficial Ca^{2+} binding site of low affinity.

Keywords Cytochrome *c* peroxidase · Ca^{2+} binding sites · Cytochrome c_{551} · Electron transfer

Abbreviations *CCP*: cytochrome *c* peroxidase · *DAD*: diaminodurol (2,3,5,6-tetramethyl-*p*-phenylenediamine) · *EGTA*: ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid · *ICP*: inductive coupled plasma emission analysis · *Pa*: *Paracoccus* · *Ps*: *Pseudomonas* · *vvm*: volume of air per volume of media per minute

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Introduction

Hydrogen peroxide is produced by cells as a result of an incomplete reduction of oxygen during their metabolism. Owing to the toxicity of this molecule, a detoxification process is needed in order to maintain viability of the cell. One of these defense mechanisms involves the reduction of hydrogen peroxide to water by a peroxidase, which draws electrons from the electron transport chain.

Bacterial peroxidases are heme proteins. Well-characterized members of this group are the cytochrome *c* peroxidases (CCP) from *Pseudomonas* (*Ps*.) *aeruginosa* [1, 2, 3, 4], *Paracoccus* (*Pa*.) *denitrificans* [5, 6, 7], *Ps. nautica* [8], *Nitrosomonas europaea* [9] and *Methylococcus capsulatus* [10]. In these enzymes, one of the hemes has a high redox potential, reflecting a histidine-methionine coordination, and is believed to function as the

electron transfer heme. The second heme is the peroxidic center, with a low redox potential and a bis-histidine coordination in the oxidized form [11].

During catalysis, CCP can accept two electrons from ferrocytochrome *c* according to the following equation:

$$H_2O_2 + 2H^+ + 2\text{Cyt } c(Fe^{2+}) \rightarrow 2H_2O + 2\text{Cyt } c(Fe^{3+}) \quad (1)$$

Villalain and co-workers [12] identified a CCP in the denitrifying bacteria *Ps. stutzeri*, which was shown to have a M_r of 37,000 (as determined by SDS-PAGE). One of the hemes of this enzyme could be reduced by the addition of sodium ascorbate (like the high-potential heme of the CCP of *Ps. aeruginosa*). From spectroscopic studies it was proposed that the high-potential heme was low-spin in all three redox states and the low-potential heme was in a high-spin/low-spin equilibrium in the fully oxidized enzyme.

From studies of the enzyme from *Ps. aeruginosa* [13] it is now known that the high-potential heme is in a high-spin/low-spin equilibrium in the oxidized enzyme and the low-potential heme is in a low-spin configuration. Reduction of the high-potential heme to give the mixed valence enzyme leads to a fully low-spin configuration at this heme but a switch to a high-spin state at the low-potential heme.

In the case of the *Pa. denitrificans* and *Ps. nautica* enzymes, the switch from low-spin to high-spin configuration at the low-potential heme requires the presence of Ca^{2+} [8, 14, 15], a requirement not investigated in the *Ps. aeruginosa* enzyme.

The crystal structure of the oxidized form of the CCP from *Ps. aeruginosa* [11] shows an enzyme of two domains, each with one covalent heme *c*. A Ca^{2+} binding site is present at the interface of the two domains.

In the more complex case of the *Pa. denitrificans* enzyme, Gilmour et al. [16] proposed that this site (which they called site I) is also present but that there is a second Ca^{2+} binding site (called site II) which is mostly empty in the isolated enzyme and the re-occupancy of which is required for the switch to the high-spin state and enzyme activity.

In this paper we address the differences in Ca^{2+} dependence shown by the different bacterial peroxidases and the different electrostatic considerations that govern their interaction with donor cytochromes.

Materials and methods

Growth of the cells

Ps. stutzeri (ATCC 11607) was grown at 30 °C for 23 h in a medium containing trisodium citrate (15 g/L), NaCl (5 g/L), $MgCl_2{\cdot}7H_2O$ (12.3 g/L), KCl (0.75 g/L), Tris (6.05 g/L), NH_4Cl (1 g/L), $CaCl_2{\cdot}2H_2O$ (1.49 g/L), yeast extract (1 g/L), $FeSO_4$ (2 mg/L), $K_2HPO_4{\cdot}3H_2O$ (18.6 mg/L) and a Starkey oligoelement solution (0.2 mL/L) [17]. The Starkey oligoelement solution, $FeSO_4$ solution and $K_2HPO_4{\cdot}3H_2O$ solution were sterilized separately and added to the culture medium after its sterilization. The reactor (Biolafitte, Poissy, France) had a working volume of 30 L. The aeration rate was adjusted to 0.2 vvm (volume of air per volume of media per minute) and the agitation speed to 150 rpm (using a central axis with flat-blade turbines). To monitor the pH, a Mettler-Toledo pH electrode (Greifensee, Switzerland) was used. The pH was kept near 7 by titration with a 4 M HCl solution. Cells were harvested at the beginning of the stationary growth phase, before the oxygen rose to detectable levels as measured by a polarographic oxygen probe (Biolafitte). Oxygen concentration falls soon after the inoculation of the culture medium to a level below the detection limit of the probe.

Purification of CCP

Cells were harvested by centrifugation in a Beckman centrifuge, model J-6B in a JS 5.2 rotor, at 5000 rpm for 25 min at 4 °C and washed with 10 mM sodium phosphate buffer, pH 7. Spheroplasts were prepared according to Goodhew et al. [5]. Protease inhibitors were added (10 mM benzamidine and 1 mM phenylmethanesulfonyl fluoride) to the supernatant (periplasm).

All purification steps were performed continuously at 4 °C. The supernatant obtained as described above was loaded onto a DEAE-52 column, equilibrated with 10 mM Tris-HCl buffer, pH 8. Elution was performed by application of a linear gradient between 10 mM Tris-HCl (pH 8) and 400 mM NaCl + 10 mM Tris-HCl (pH 8). CCP was eluted at 250 mM NaCl, concentrated on a small DEAE-52 column equilibrated with 10 mM Tris-HCl (pH 8) and stripped with 10 mM Tris-HCl (pH 8) + 500 mM NaCl. The eluate was then applied to a Sephadex G 150-50 molecular exclusion column (Amersham Biosciences, 3×83 cm) equilibrated with 20 mM Tris-HCl (pH 8) + 100 mM NaCl. The fraction containing CCP was concentrated in a Vivaspin-4 apparatus (Vivascience), with a 10,000 M_r cut-off membrane. The resultant fraction was applied onto a hydroxyapatite column (Bio-Rad, 3×7.5 cm), equilibrated with 1 mM sodium phosphate + 100 mM NaCl (pH 7). Elution was performed by application of a linear gradient between 1 mM sodium phosphate (pH 7) + 100 mM NaCl and 100 mM of sodium phosphate buffer (pH 7). CCP eluted at 80 mM sodium phosphate and 20 mM NaCl. The buffer was exchanged to 10 mM Hepes (pH 7.5) and the protein was stored frozen at –70 °C.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SDS-PAGE was performed as described by Weber and Osborn [18] and heme staining according to Goodhew et al. [19].

Purification of cytochrome c_{551}

The fraction containing cytochrome c_{551} from the DEAE-52 column, after concentration in a Diaflo apparatus with a YM 3 membrane, was applied onto a Superdex 75 molecular exclusion column (Amersham Biosciences, 2.6×55 cm) equilibrated with 0.3 M Tris-HCl buffer (pH 7.6). The cytochrome c_{551} fraction was concentrated in a Diaflo apparatus with a YM 3 membrane. Ionic strength was lowered by dilution and the sample was loaded onto a Q-Resource cartridge (Amersham Biosciences, 6 mL), equilibrated with 10 mM Tris-HCl (pH 7.6). Pure cytochrome c_{551} eluted at 75 mM Tris-HCl, using a gradient between 10 and 500 mM Tris-HCl (pH 7.6). The buffer was exchanged to 10 mM Hepes (pH 7.5) and the protein was stored frozen at –70 °C. The A_{410}/A_{275} ratio of pure cytochrome *c* was 4.5.

Spectroscopic methods

UV-visible spectra were recorded in a Shimadzu UV-265 split-beam spectrophotometer connected to an IBM PC compatible computer.

Low-temperature EPR measurements were made using an X-band Brucker EMX spectrometer equipped with an Oxford Instruments liquid helium flow cryostat.

Enzymatic assays were performed in a Diode Array 8452A Hewlett Packard spectrophotometer equipped with hydraulic powered magnetic stirring.

Enzymatic assay

The enzymatic assay was performed at room temperature, as described by Gilmour et al. [20], by monitoring the oxidation of the reduced electron donor cytochrome *c* at 550 nm. Sodium ascorbate (1 mM) was used to reduce cytochrome *c*, which was then applied to a Sephadex G-25 (PD 10, Amersham Biosciences) column equilibrated in 10 mM Hepes buffer (pH 7.5) to remove the excess of reducing agent. The CCP stock solution was diluted in 10 mM Hepes buffer (pH 7.5) to a final concentration of 4 μM and incubated for 30 min at room temperature in the presence of ascorbate (1 mM) and diaminodurol (DAD, 10 μM). When required, Ca^{2+} (1 mM) was added to this solution. Catalytic center activity was defined as in Gilmour et al. [20].

The assay was always started by addition of the enzyme to the reaction mixture (with a total volume of 2 mL). The kinetics is pseudo-first-order. Two cytochromes were used as electron donors: cytochrome c_{551} from *Ps. stutzeri* and horse heart cytochrome *c* (Sigma).

Protein and metals determination

Protein concentration was determined according to Lowry et al. [21]. Iron and Ca^{2+} contents were determined by inductive coupled plasma emission analysis (ICP, Thermo Jarrel-Ash 965, Chemical Analyses Laboratory, University of Georgia).

Molecular mass determination

Sedimentation velocity experiments were carried out in an Optima XL-A (Beckman, Fullerton, Calif.) analytical ultracentrifuge fitted with an absorbance optical system. 400 μL each of sample and solvent were filled into their respective channels in 12 mm optical pathlength ultracentrifuge cells and run at 45,000 rpm. Movement of the boundary was monitored at a wavelength of 440 nm. Data were captured using the manufacturer's software (Microcal Origin v. 4.1) and analysed using the DCDT+ software developed by Philo [22].

Results

Cell growth and optimization of CCP production in *Ps. stutzeri*

To optimize the CCP production we carried out several batch growths in different media. Lactate, dextrose, citrate and acetate were used as carbon and energy sources in a minimum medium (see Materials and methods). Rich media, such as nutrient broth and yeast malt, were also used. The highest yield of CCP was obtained when citrate was present at 15 g/L, whereas at concentrations higher than 20 g/L the cell growth was inhibited. Under the optimum conditions, 6 g wet cell weight was obtained per liter of culture.

The aeration of the medium, and the concentration of dissolved oxygen, were found to be important factors which influence the yield of CCP. With an aeration of 1 vvm, CCP is not expressed at detectable levels, while with 0.2 vvm, good yields of CCP were obtained, but only when cells were harvested before the oxygen level rose.

If the pH is allowed to rise during growth (final pH 8.4), a lower cell yield is obtained but the cells contain a higher concentration of CCP (and lower concentration of cytochrome c_{551}).

Purification of CCP

The A_{408}/A_{275} ratio of the pure CCP was 4.4. This sample gave a single band in SDS-PAGE gels stained with Coomassie Blue and a heme staining. Other known *Pseudomonas* cytochromes can be identified in the profile of the initial DEAE cellulose chromatography. These cytochromes were partially purified and the identification confirmed by examination of their visible spectra. The purity of the CCP preparation was also checked using chromatography on a Phenomenex Biosep DEAE-p anion exchange column (results not shown). Although the CCP gave a complex pattern in the absence of Ca^{2+}, inclusion of Ca^{2+} in the elution buffer simplified the elution pattern to a single peak.

ICP determination of the Ca^{2+} content of purified CCP gave 1.5 ± 0.3 Ca^{2+} per dimer.

Molecular mass

The sedimentation velocity profiles of CCP centrifuged in the presence of Ca^{2+}, in the presence of ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), and without Ca^{2+} or EGTA, were analysed by the DCDT+ method [22], giving the sedimentation coefficient distributions shown in Fig. 1. The $s_{20,w}$ value of the CCP from *Ps. stutzeri* in the absence of added Ca^{2+} (4.67 S) is very similar to that of *Pa. denitrificans* CCP in the presence of Ca^{2+} (4.78 S; G.W. Pettigrew, unpublished results), a value consistent with the dimer (theoretical value of 75,026 for the *Pa denitrificans* dimer). Sedimentation of the *Ps. stutzeri* enzyme in the presence of EGTA yields a $s_{20,w}$ value (2.83 S) which is very similar to that obtained for the *Pa. denitrificans* enzyme in the absence of added Ca^{2+}. The corresponding M_r of 36,000 [23] is consistent with a monomeric state for each enzyme (theoretical M_r of 37,513 for the *Pa. denitrificans* enzyme). Sedimentation of the *Ps. stutzeri* enzyme in the presence of added Ca^{2+} shifts the $s_{20,w}$ value to 5.58 S. This indicates aggregation of this enzyme beyond the dimer but the $g(s^*)$ distribution was complex and could not be fitted to a single species.

Molecular exclusion chromatography cannot be used to obtain reliable M_r for non-spherical molecules such as the monomer and dimer of CCP. However, the method can indicate changes in aggregation. The results of Fig. 2 show the elution profile of the *Ps. stutzeri* enzyme under different conditions. The untreated enzyme elutes with an apparent M_r of 67,000. This is consistent with the dimeric state observed by ultracentrifugation. However, the peak is complex, with a trailing edge, and this may be due to partial monomerization as the protein passes down the

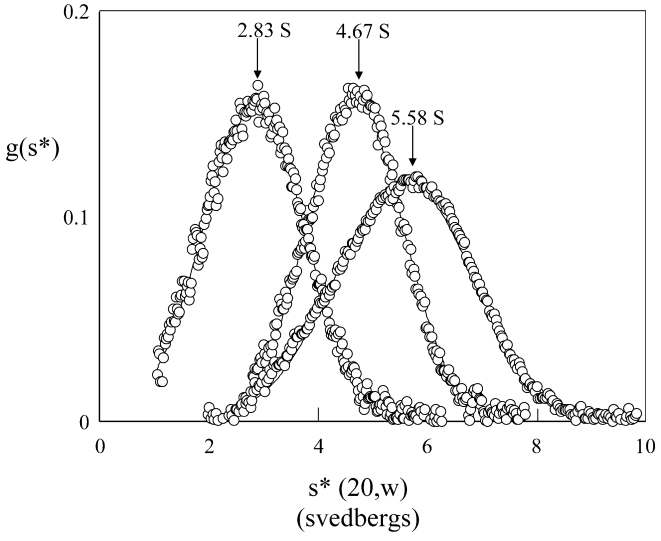

Fig. 1 Sedimentation velocity ultracentrifugation of *Ps. stutzeri* CCP. Paired scans within a set of nine sequential scans were used to produce a distribution of sedimentation coefficients using the DCDT+ method of Philo [22] and Stafford [28]. Sedimentation velocity experiments were carried out at 45,000 rpm, at 25 °C, in a Optima XL-A (Beckman) analytical ultracentrifuge. The progress of the boundary was monitored at 440 nm. *Ps. stutzeri* CCP (10 μM) in 10 mM Hepes (pH 7.5) was centrifuged in the presence of 1 mM EGTA (2.83 S), in the presence of 2 mM Ca^{2+} (5.58 S) and in the presence of neither (4.67 S)

column. In the presence of EGTA an apparent M_r of 51,000 was obtained. This was also obtained for the *Pa. denitrificans* enzyme (see Fig. 3) and reflects the monomeric state detected by ultracentrifugation. The discrepancy with the theoretical M_r of the monomer is due to the known elongated structure of the monomer. For the *Ps. stutzeri* enzyme, in the presence of added Ca^{2+} or at higher protein concentrations there is a shift to higher apparent M_r (80,000 and 87,000, respectively). This phenomenon was also seen in the ultracentrifugation experiments, but is not seen with the *Pa. denitrificans* enzyme which elutes with an apparent M_r of 74,000 in the presence of Ca^{2+}, a value entirely consistent with the dimer.

Thus the results of the molecular exclusion chromatography and the ultracentrifugation are in agreement. The *Ps. stutzeri* enzyme is already dimeric in the untreated state. Added Ca^{2+} or higher protein concentrations partially shift the enzyme to a higher state of aggregation, presumably tetramer.

UV-visible spectroscopy

The spectrum of the isolated CCP shows a Soret maximum at 408 nm and a weak absorption band at 630 nm, which, in other bacterial peroxidases, has been shown to be due to a high-spin/low-spin equilibrium at the high-potential heme (Fig. 4A). The absorbance at 408 nm was related to the iron content of the solution determined by ICP and an extinction coefficient of 252 mM^{-1} cm^{-1} was obtained assuming two iron atoms per monomer.

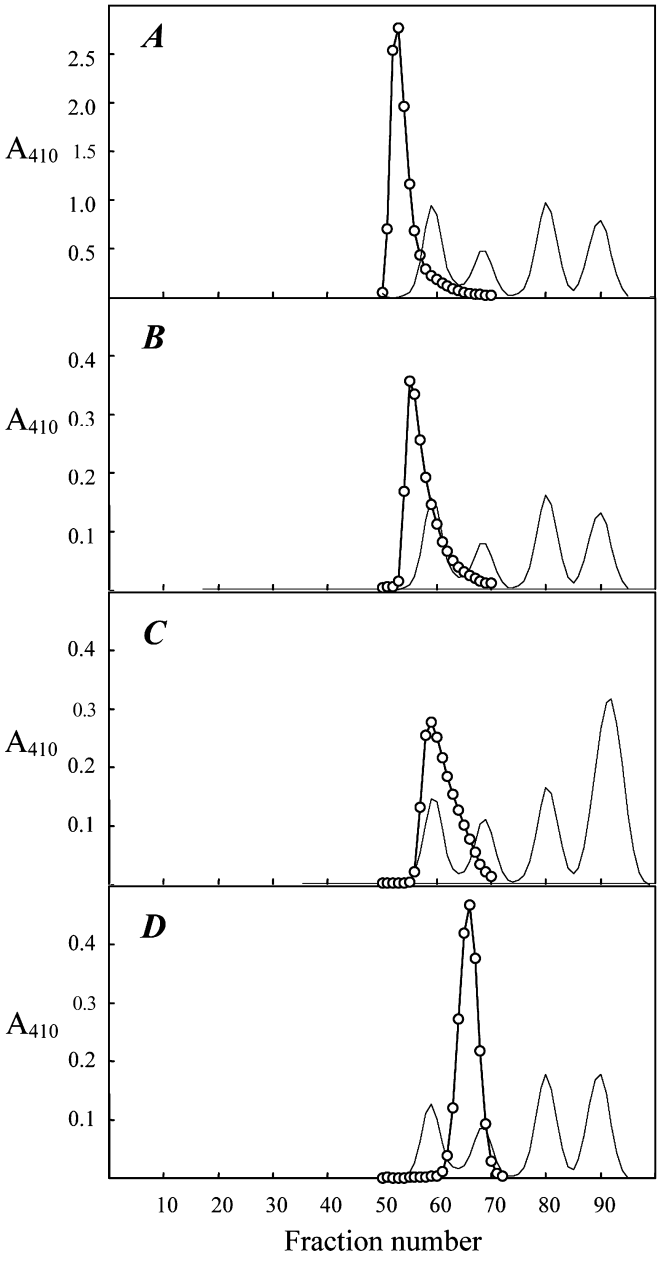

Fig. 2A–D Molecular exclusion chromatography of *Ps. stutzeri* CCP. CCP from *Ps. stutzeri* was subjected to chromatography at 4 °C on a column of Sephadex G150-50 (2.2×84 cm) in 20 mM Tris-HCl (pH 8.0) + 100 mM NaCl. **A** 175 nmol CCP in 2 mL column buffer containing 2 mM Ca^{2+}; **B** 30 nmol CCP in 2 mL column buffer containing 2 mM Ca^{2+}; **C** 30 nmol CCP in 2 mL column buffer; **D** 30 nmol CCP in 2 mL column buffer containing 1 mM EGTA. In each case the column was equilibrated with a buffer of the same composition as that of the loading solution. The elution of the CCP was monitored by measurement of the absorption at 410 nm. A set of standard proteins (serum albumin 66 kDa, ovalbumin 45 kDa, carbonic anhydrase 29 kDa and myoglobin 17 kDa) were run separately for each solution composition and their elution positions are indicated by the *faint lines*. Apparent M_r values were estimated from a plot of the logarithm of the M_r of these standard proteins versus elution volume

Upon reduction with sodium ascorbate the Soret maximum is shifted to 418 nm and a shoulder at 408 nm remains, corresponding to the non-reduced second

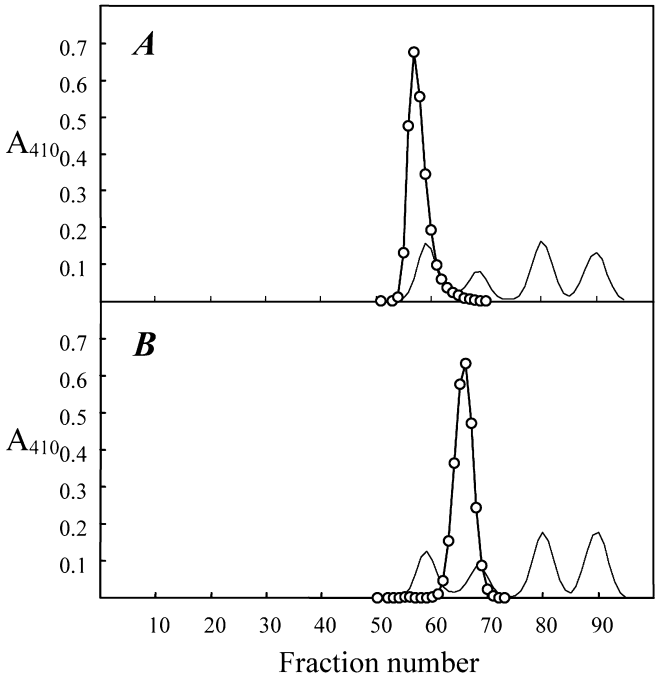

Fig. 3A, B Molecular exclusion chromatography of *Pa. denitrificans* CCP. CCP from *Pa. denitrificans* was subjected to chromatography at 4 °C as for Fig. 2. **A** 30 nmol CCP in 2 mL column buffer containing 2 mM Ca^{2+}; **B** 30 nmol CCP in 2 mL column buffer containing 1 mM EGTA

heme. The α and β bands appear at 556 nm and 524 nm, respectively. Reduction is completed in 1 min. A weak absorption band is observed at 630 nm, distinct in appearance from that seen in the oxidized form. Addition of Ca^{2+} does not induce any further changes to the spectrum (Fig. 4A). This behavior is distinct from that seen in the *Pa. denitrificans* enzyme at pH 7.5, where there is no high-spin band after reduction with ascorbate and this band appears only on addition of Ca^{2+} [14]. When reduction follows treatment of the oxidized enzyme with EGTA, high-spin features at 380 nm and 630 nm are formed only upon addition of Ca^{2+} (Fig. 4B). Remarkably, however, the enzyme reduced with ascorbate in the presence of EGTA does slowly develop a high-spin band at 380 nm over a period of time (Fig. 5A). The high-spin development is simultaneous to peroxidase activity recovery (Fig. 5B).

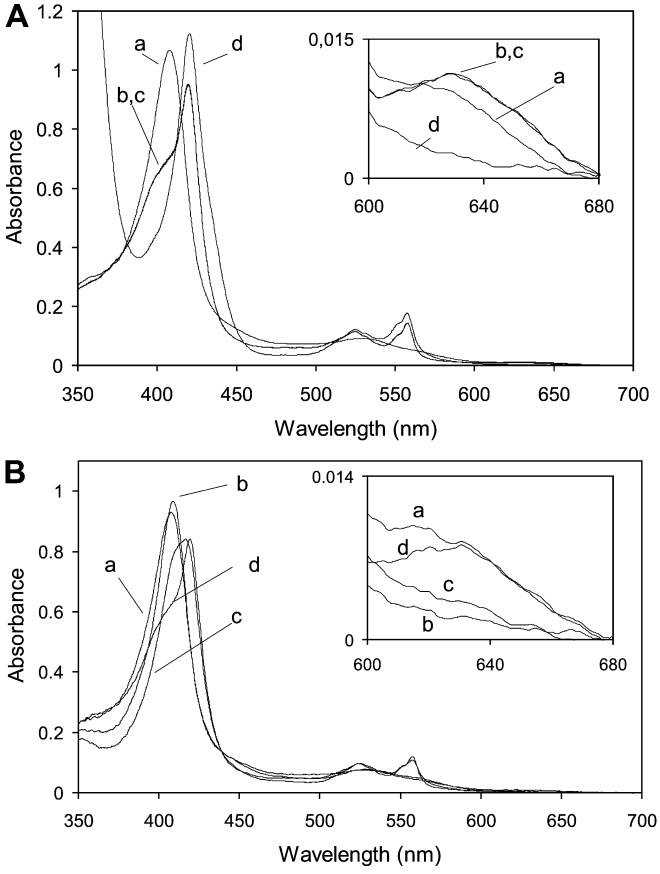

Fig. 4A, B UV-visible absorption spectra of *Ps. stutzeri* CCP (4 μM) in 10 mM Hepes buffer (pH 7.5). The *insert* is an amplification of the 630 nm band region. **A** *a*, oxidized protein; *b*, mixed valence protein obtained by treatment with sodium ascorbate (1 mM) and DAD (10 μM) and incubation for 15 min; *c*, mixed valence protein obtained as in *b* and then incubated with Ca^{2+} (free Ca^{2+} concentration of 1 mM) for 30 min; *d*, fully reduced protein obtained by treatment with sodium dithionite. **B** Spectra of the protein treated with EGTA: *a*, oxidized protein; *b*, oxidized protein after treatment with EGTA (1 mM) for 30 min; *c*, mixed valence protein obtained by treatment of *b* with ascorbate (1 mM) and DAD (10 μM) and incubated for 1 min; *d*, mixed valence protein obtained as in *c* and then incubated with Ca^{2+} (1 mM) for 30 min

Enzymatic activity

Effect of the electron donor concentration on the enzymatic activity

With both electron donors used – *Ps. stutzeri* cytochrome c_{551} and horse heart cytochrome *c* – the curves obtained for the catalytic center activity versus electron donor concentration obey apparent Michaelis-Menten kinetics.

The values obtained for the maximum catalytic center activity and concentration at half-maximum turnover are $(20.3 \pm 0.9) \times 10^3$ min^{-1} and 54 ± 4 μM for cytochrome c_{551} and $(11.7 \pm 0.6) \times 10^3$ min^{-1} and 11.8 ± 1.3 μM for horse heart cytochrome *c*, respectively. These values are based on a non-linear regression of the Michaelis-Menten equation. However, owing to limitations of the method, the substrate concentration range for the cytochrome c_{551} falls below the very high K_m value and the K_m estimate must be regarded as approximate.

Effect of hydrogen peroxide concentration on the enzymatic activity

A plot of the catalytic center activity versus hydrogen peroxide concentration yielded a maximum catalytic center activity of $(5.3 \pm 0.1) \times 10^3$ min^{-1} and a hydrogen peroxide concentration at half-maximum turnover of 1.8 ± 0.05 μM. These results show that saturation is

already achieved at the hydrogen peroxide concentration used (18 μM) in the other enzymatic assays described, thus validating the use of pseudo-first-order kinetics as in Gilmour et al. [20].

Effect of reduction by ascorbate and incubation with Ca^{2+} on enzyme activity

The results of these assays differ with the two electron donors used. In the case of the *Pa denitrificans* CCP, pre-incubation with Ca^{2+} was required to activate the enzyme [20]. In the case of the CCP from *Ps stutzeri*, pre-incubation with Ca^{2+} has no effect on activity (Table 1). However, the presence of Ca^{2+} in the assay solution inhibits the oxidation of horse cytochrome *c* but not cytochrome c_{551}.

The same pattern of results was obtained at pH 6 with each electron donor.

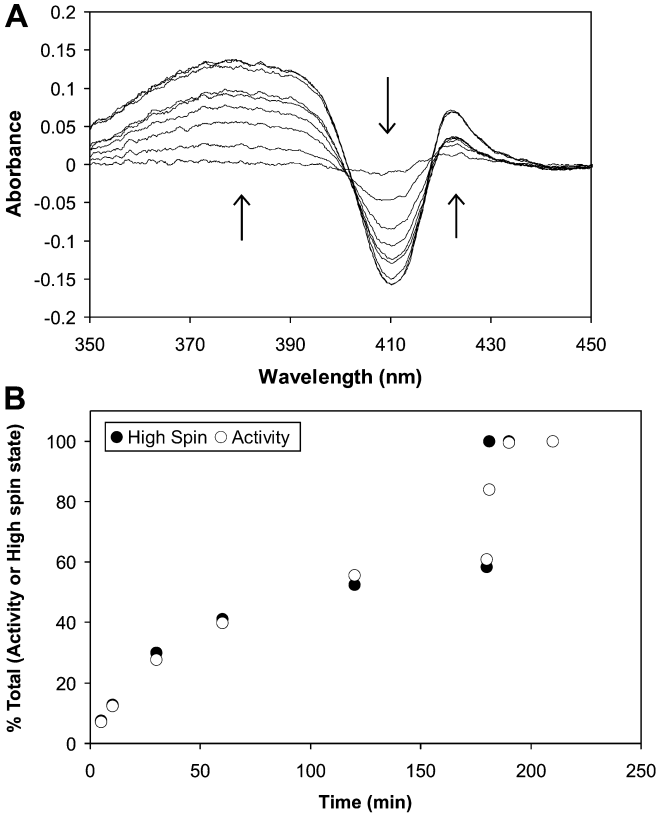

Fig. 5 **A** Difference spectra of the mixed valence CCP after treatment with EGTA (1 mM) in the oxidized form minus the spectrum of the mixed valence protein (after 1 min incubation). The *arrows* indicate increasing timepoints after reduction. The three last difference spectra are after 2 mM Ca^{2+} addition. **B** The time course of appearance of the high-spin state (as measured by the absorbance change at 380 nm in **A**) and enzyme activity after EGTA treatment (measured as described in Materials and methods) and after ascorbate reduction

Effect of the ionic strength on the enzymatic activity

The effect of the ionic strength on the enzymatic activity of CCP with cytochrome c_{551} as electron donor was different from that obtained with the horse heart cytochrome *c* and also from the effect of ionic strength on the reactivity of *Pa. denitrificans* CCP [24]. With cytochrome c_{551}, the catalytic center activity increases with ionic strength (Fig. 6). With horse heart cytochrome *c*, the catalytic center activity decreases exponentially.

EPR spectroscopy

The EPR spectra of the oxidized enzyme, the mixed valence enzyme and the mixed valence enzyme incubated with Ca^{2+} are shown in Fig. 7. In the oxidized state (Fig. 7A), three sets of signals can be observed. By analogy with the results for the *Ps. aeruginosa* and *Pa. denitrificans* enzymes [13, 14], the resonances at $g_{max} = 3.00$, $g_{med} = 2.28$ and $g_{min} = 1.34$ can be attributed to the low-potential heme, while resonances at $g_{max} = 3.39$ and $g_{med} = 2.03$ are attributed to the high-potential heme (with an estimated g_{min} equal to 0.6). It is known that, in these related bacterial enzymes, the high-potential heme is in a high-spin/low-spin equilibrium. In the *Ps. stutzeri* CCP a third set of resonances with *g* values characteristic of a high-spin heme system ($g = 5.7$ and $g = 5.15$) can be observed in the low-temperature spectrum. These resonances can be assigned to the high-spin form of the high-potential heme, which was seen also by visible spectroscopy. The set of weak signals observed between $g = 4.8$ and 3.7 is probably due to a minor ferric impurity. Thus the low-spin features of the spectrum can be simulated in the spectrum of Fig. 7B by the contribution of two hemes.

Table 1 The effect of incubation of the enzyme with Ca^{2+} and the presence of Ca^{2+} in the assay on the enzymatic activity of the *Ps. stutzeri* CCP. Activity was assayed in 10 mM Hepes buffer (pH 7.5), 7 μM of electron donor cytochrome *c*, 20 nM CCP and 18 μM H_2O_2. The numbers in parentheses are estimated standard deviations in the last significant digits

CCP stock	Catalytic center activity (min^{-1})			
	With *Ps. stutzeri* cytochrome c_{551}		With horse heart cytochrome *c*	
	Ca^{2+} in assay (1 mM)	No Ca^{2+} in assay	Ca^{2+} in assay (1 mM)	No Ca^{2+} in assay
Mixed valence, incubated with Ca^{2+}	4600 (400)	4440 (600)	3390 (50)	5350 (340)
Mixed valence	4540 (60)	4480 (400)	3310 (70)	5940 (200)

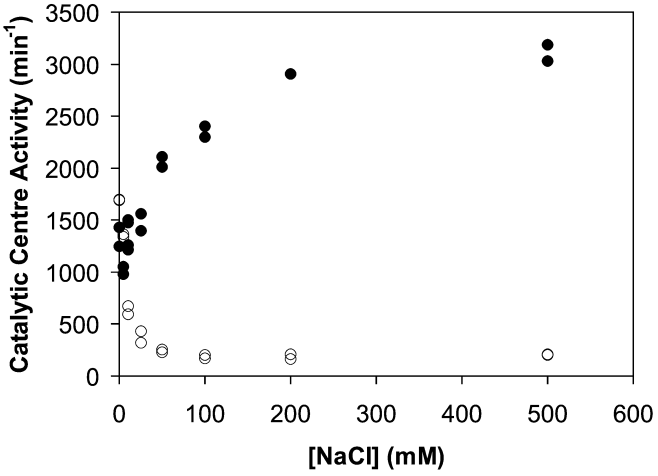

Fig. 6 Effect of the ionic strength on the enzymatic activity with the two ferrocytochromes used as electron donors. *Filled circles*: *Ps. stutzeri* cytochrome c_{551}; *open circles*: horse heart cytochrome *c*. Activity was assayed in 10 mM Hepes buffer (pH 7.5), 20 nM CCP, 18 μM H_2O_2, 7 μM ferrocytochrome and the appropriate concentration of NaCl

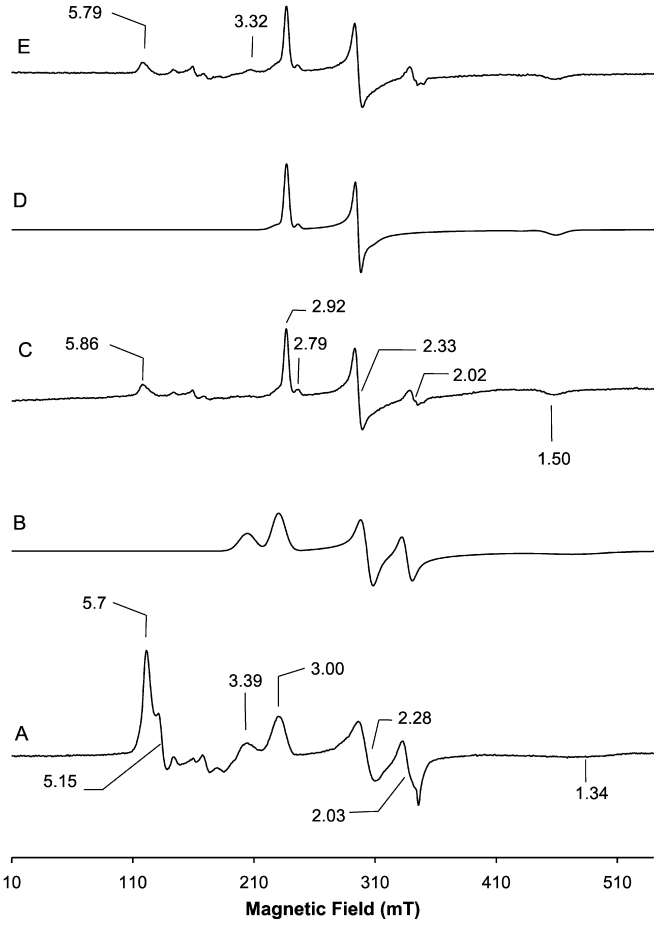

Fig. 7 EPR spectra of the CCP of *Ps. stutzeri* oxidized protein (**A**), the mixed valence protein (**C**) and the mixed valence protein incubated with Ca^{2+} (2 mM Ca^{2+}) (**E**). Experimental conditions: microwave frequency, 9.65 GHz; temperature, 8 K; number of scans, 3; receiver gain, 2×10^5; modulation amplitude 1 mT. **B** is the simulation of the spectrum **A** for the low-spin hemes. The parameters used in the simulation were for low-potential heme: $g_{max}=2.99$, $g_{med}=2.27$ and $g_{min}=1.44$; and for high-potential heme: $g_{max}=3.37$, $g_{med}=2.05$ and $g_{min}=0.61$. **D** is the simulation of the spectrum **C** for the low-spin hemes. The parameters used in the simulation were for species I: $g_{max}=2.91$, $g_{med}=2.33$ and $g_{min}=1.50$; and for species II: $g_{max}=2.80$, $g_{med}=2.30$ and $g_{min}=1.60$. As there is still a minor contribution of non-reduced high-potential heme, the parameters used to simulate this contribution are the same as simulation **B**

Upon incubation with ascorbate and DAD (20 min under an argon atmosphere, see Fig. 7C) the signals of the high-potential heme are lost, as expected from its redox properties. The remaining resonances must therefore be due to the low-potential heme, which remains in the ferric state. However, the signal is sharper and has different *g* values when compared to the one obtained for the oxidized form of the enzyme, reflecting a subtle heme-heme interaction. Contrary to what was observed in *Pa. denitrificans* [25], only a major set of resonances, $g=2.92$, 2.33 and 1.50, is observed. This set is comparable to the so-called species I of *Pa. denitrificans*, and only a minor resonance at $g=2.79$ hints at the presence of species II. Incubation of the mixed valence enzyme with Ca^{2+} has no observable effect on the EPR spectrum (Fig. 7E). This finding is consistent with the other spectroscopic evidence which shows that the Ca^{2+} is already bound to *Ps. stutzeri* CCP and species I represents the active form of low-potential heme, which at room temperature is in the high-spin state. This single EPR-visible heme is simulated in Fig. 7D. Missing from the simulation are the weak high-spin heme signals still present in the mixed valence spectra (Fig. 7C and E, $g=5.7$–5.9). These may be due either to the presence of some non-reducible protein or to the presence of some high-spin state at the low-potential heme even at this low temperature.

Discussion

Two very important factors in the optimization of the production of CCP from *Ps. stutzeri* were the composition of the growth medium and the rate of aeration. Citrate was found to be the best carbon and energy source to optimize the production of CCP. Cytochrome c_{551} also showed higher levels of production when the bacterium was grown on this medium. The best aeration rate for the production of CCP was 0.2 vvm. Cytochrome c_{551} also gave good yields at this aeration rate.

Purification of CCP has to be a very fast operation in order to prevent proteolysis. Freezing intermediate fractions during the purification process results in a less active CCP preparation. SDS-PAGE analysis of these preparations revealed proteolysis products of approximately 10, 16 and 28 kDa.

Spectroscopic results of Fig. 4A indicate that CCP from *Ps. stutzeri* does not need added Ca^{2+} ions for high-spin formation in the mixed valence state. Ascorbate reduction of CCP treated with EGTA results in a

CCP that does not show, in difference spectra, the appearance of the 380 nm band used as a marker for high-spin formation. Remarkably, however, the enzyme reduced with ascorbate in the presence of EGTA does slowly develop these high-spin spectroscopic features over a period of time (Fig. 5A). Sixty percent of the 380 nm band in difference spectra appears with no additional Ca^{2+}. This high-spin appearance with incubation time is closely matched by the appearance of enzyme activity (Fig. 5B). Thus, unlike the *Pa. denitrificans* enzyme [14], the *Ps. stutzeri* CCP appears to be purified with a tightly bound Ca^{2+} involved in the conversion to the active high-spin state. Although this Ca^{2+} can be removed by EGTA from the oxidized enzyme, subsequent reduction of the enzyme leads to a Ca^{2+} binding site that is of such high affinity that it can regain the Ca^{2+} from the EGTA (Fig. 5). At this pH (7.5), the apparent dissociation constant of EGTA for Ca^{2+} is 38 nM and thus the protein site competes effectively with this high affinity binding.

The EPR results are also consistent with the conclusion that the CCP from *Ps. stutzeri* already contains a tightly bound Ca^{2+}. Both of the molecular weight determinations (by ultracentrifugation and on a calibrated gel filtration column) were consistent with a Ca^{2+} site involved in dimerization that is already filled in the purified CCP.

An already occupied Ca^{2+} binding site in the *Ps. stutzeri* enzyme is also consistent with the kinetic measurements of Table 1. Pre-incubation of the enzyme with Ca^{2+} has no effect on the activity with either cytochrome substrate. On the other hand, the presence of Ca^{2+} in the assay solution is inhibitory for the oxidation of horse cytochrome *c* but not for the physiological donor, cytochrome c_{551}. We propose that this inhibitory effect is due to a charge repulsion effect of bound positive Ca^{2+} ions at the surface of the CCP towards the positively charged horse heart cytochrome *c*. In contrast, both the cytochrome c_{551} and the CCP itself have acidic isoelectric points and will carry a net negative charge at the pH of the assay. Such a loose binding of Ca^{2+} at the surface of the *Ps. stutzeri* CCP is also consistent with the higher order aggregation beyond the dimeric state that was observed in both ultracentrifugation and molecular exclusion experiments in the presence of added Ca^{2+}.

The effect of the association of like-charged molecules is strikingly seen in the ionic strength dependence of the activity (Fig. 6). With the cytochrome c_{551} as donor, the enzyme activity increases with increasing ionic strength, which probably reflects the shielding of the intrinsic charge repulsion by salt ions. It may also reflect an interface that is dominated by hydrophobic interactions that are enhanced at higher salt concentration. In contrast, with horse heart cytochrome *c* as donor, the fall in activity with increasing ionic strength is indicative of the interaction of two oppositely charged molecules [26], as seen for *Pa. denitrificans* and its electron donors. Interestingly, however, we do not observe the initial increase in activity with ionic strength in the low ionic strength region that is observed for the *Pa. denitrificans* enzyme [24]. In the latter this was interpreted as due to the formation of too tight a binding at very low ionic strength, which either prevents the mobility within the encounter complex needed for fast electron transfer [27] or leads to slow dissociation and inhibition of turnover. We suggest that the non-physiological donor, horse cytochrome *c*, may not be able to form many strong specific interactions on the *Ps. stutzeri* CCP surface and thus may not be susceptible to these rate-limiting effects.

In conclusion, our results are consistent with a single tight Ca^{2+} binding site in *Ps. stutzeri* CCP which is occupied in the isolated enzyme and which is required for dimer formation and the transition to the active high-spin state at the peroxidatic heme. In addition, there is evidence for a surface site, which affects the kinetics of oxidation of cytochrome and higher order aggregation.

Since we observe 0.75 mol Ca^{2+} bound to the monomer in the isolated enzyme, we propose that this tightly bound Ca^{2+} corresponds to the Ca^{2+} that sits between the heme groups in the X-ray structure of the *Ps. aeruginosa* CCP [11].

The *Pa. denitrificans* CCP is also isolated with a tightly bound Ca^{2+} (called site I). However, occupancy of that site is not sufficient for either dimer formation or transition to the active high-spin state at the peroxidic heme. For these changes to occur, a second Ca^{2+} must be bound to a relatively weak binding site (called site II).

Thus, the main difference between the two enzymes is in the binding affinity of the Ca^{2+} site responsible for activity and dimerization. This is high affinity in the oxidized *Ps. stutzeri* enzyme and low affinity in its *Pa. denitrificans* counterpart. We propose that the *Ps. aeruginosa* enzyme will resemble the *Ps. stutzeri* enzyme in this respect.

Further characterization of CCP from *Ps. stutzeri* and the cytochrome c_{551} is currently being done to elucidate the nature of intermolecular electron transfer and the catalytic mechanism.

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