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True wild type and recombinant wild type cytochrome *c* oxidase from *Paracoccus denitrificans* show a 20-fold difference in their catalase activity

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ABSTRACT

The four subunit (SU) aa_3 cytochrome *c* oxidase (CcO) from *Paracoccus denitrificans* is one of the terminal enzymes of the respiratory chain. Its binuclear active center, residing in SU I, contains heme a_3 and Cu_B. Apart from its oxygen reductase activity, the protein possesses a peroxidase and a catalase activity. To compare variants and the wild type (WT) protein in a more stringent way, a recombinant (rec.) WT strain was constructed, carrying the gene for SU I on a low copy number plasmid. This rec. WT showed no difference in oxygen reductase activity compared to the American Type Culture Collection (ATCC) WT CcO but surprisingly its catalase activity was increased by a factor of 20. The potential over-production of SU I might impair the correct insertion of heme a_3 and Cu_B because of a deficiency in metal inserting chaperones. An altered distance between heme a_3 and Cu_B and variations in protein structure are possible reasons for the observed increased catalase activity. The availability of chaperones was improved by cloning the genes *ctaG* and *surf1c* on the same plasmid as the SU I gene. The new rec. WT CcO showed in fact a reduced catalase activity. Using differential scanning calorimetry no significant difference in thermal stability between the ATCC WT CcO and the rec. WT CcO was detected. However, upon aging the thermal stability of the rec. WT CcO was reduced compared to that of the ATCC WT CcO pointing to a decreased structural stability of the rec. WT CcO.

1. Introduction

The four subunit (SU) aa_3 cytochrome *c* oxidase (CcO) from the soil bacterium *Paracoccus denitrificans* is one of the terminal enzymes of its aerobic respiratory chain. It catalyzes the reduction of molecular oxygen to water using electrons from cytochrome *c*. In the reaction cycle four protons are taken up from the cytoplasmic side and four additional protons are translocated ("pumped") from the cytoplasmic to the periplasmic side of the membrane. Electrons from cytochrome *c* at the periplasmic side are transferred to Cu_A (located in SU II) and then via heme *a* (located in SU I) to the active center in SU I containing heme a_3 and Cu_B. Here oxygen reduction takes place [1-6].

In order to understand the roles of individual amino acid residues, CcO variants have been produced using deletion strains of *P. denitrificans* for homologous expression. The usage of homologous expression systems is typically not expected to lead to differences between the native wild type and recombinant "wild type" proteins. In contrast, employing heterologous expressions systems may lead to differences in the protein product, e.g. recombinant human proteins miss most post-translational modifications when they are produced in prokaryotic expression systems [7]. Here we show that even the potential over-expression of one out of four genes in a homologous expression system can lead to differences in enzymatic activity of CcO isolated from native wild type (American Type Culture Collection strain number 13543, "ATCC WT"), and from a recombinant wild type ("rec. WT").

Genes coding for the four SUs of CcO and for chaperones responsible for correct cofactor insertion are distributed among four different loci in the genome. The genes coding for SU II and III of CcO and genes coding for a farnesyltransferase (*ctaB*, synthesis of heme *a*), a chaperone for copper insertion (*ctaG*) and a chaperone for insertion of heme *a* (*surf1c*) are organized in one operon (*cta*-operon) under the control of one promoter (P1) [8,9]. *P. denitrificans* contains two genes, namely *ctaDI* and *ctaDII*, coding for SU I of CcO on different loci under the control of two different promoters. The isoform of SU I encoded by *ctaDII* is detected exclusively and was found e.g. in CcO crystals whereas *ctaDI* was shown not to be expressed at least under standard conditions [9]. The gene coding for SU IV of CcO (*ctaH*) resides in a separate locus.

To compare variants and the WT of CcO in a more stringent way, a rec. WT was created: The *P. denitrificans* strain AO1 [10], with both genes for SU I (*ctaDI* and *ctaDII*), the gene for SU N of the cbb_3 cytochrome *c* oxidase (*ccoN*) and an open reading frame (*ORF4*) deleted, was supplemented

Abbreviations: CcO, Cytochrome *c* oxidase; ATCC, American Tissue Culture Collection; WT, Wild Type; SU, Subunit; DDM, β-Dodecylmaltoside; LDAO, Lauryldimethylamine-oxide; TMPD, Tetramethylphenylene-diamine

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with the coding gene for SU I (*ctaDII*) on a low-copy number plasmid under the control of the *cta*-operon promoter P1 to regain a functional C*c*O [10].

Besides the main reaction, the reduction of oxygen to water, C*c*O is able to catalyze at least two other reactions. Orii [11] described the reduction of hydrogen peroxide by bovine heart C*c*O in the presence of cytochrome *c*, i.e. C*c*O acts as cytochrome *c* peroxidase. Orii and Okunuki discovered the other activity of C*c*O, namely its catalase activity dismutating hydrogen peroxide with a turnover number of about 100 min^{-1} [12]. Recently Konstantinov and co-workers observed second order rate constants of 171 and 1200 M^{-1} sec^{-1} for the bovine and *Rhodobacter sphaeroides* aa_3 cytochrome *c* oxidases, respectively, monitoring the decay of hydrogen peroxide using a hydrogen peroxide sensitive electrode [13]. In 2010, Sedlák et al. found a second order rate constant of 63.2 M^{-1} s^{-1} for the bovine enzyme showing a slight deviation from the data presented by Bolshakov et al. [14].

To investigate possible differences in stability between the ATCC and the rec. WT C*c*O, we used capillary differential scanning calorimetry (DSC). Haltia et al. [15] have been the first to investigate the aa_3 cytochrome *c* oxidase of *P. denitrificans* using DSC. They found two transitions, the first one assigned to the denaturation of subunit I and subunit II with a midpoint temperature of 67°. The second transition with a midpoint temperature of 46.7 °C was assigned to the denaturation of subunit III. Upon reduction of cytochrome *c* oxidase an increase of the transition midpoint temperature of subunits I and II to 76 °C was reported.

This study focuses on the catalase activity of C*c*O and the unexpected difference between the ATCC WT C*c*O and the rec. WT C*c*O in terms of catalase activity. In the rec. WT a potential excess of the plasmid encoded SU I and potential shortage of the genome-encoded chaperones may lead to inaccurate cofactor insertion. A slightly different structure of the active center might increase the probability for side reactions like the catalase reaction. We discuss the difference in structure of both wild types on the basis of differential scanning calorimetry and show that additionally expressed chaperones are able to diminish the increased catalase activity of the rec. WT C*c*O.

2. Experimental Procedures

All chemicals and enzymes were obtained from Carl Roth GmbH (Karlsruhe, Germany), Sigma-Aldrich (Taufkirchen, Germany) or Fermentas (St. Leon-Rot, Germany) in highest purity if not stated otherwise.

2.1. Cloning

Initial cloning was performed in *E. coli* DH5α [16] and GM2163 [17] using the plasmid pUP39 [18] (derivative of pBBR1MCS with a copy number of approximately 30–40 copies in *Escherichia coli* and *Bordetella bronchiseptica* [19]) carrying the gene *ctaDII*. Primers used are displayed in Table 1. The rec. WT was constructed by deleting a non-coding ORF on pUP39, creating the new plasmid pFH11 with a SacI restriction site. The plasmid pFH11 was additionally used to generate plasmids carrying either *ctaG* in combination with *ctaDII* or *surf1c* and *ctaG* together with *ctaDII*. The primers listed in Table 1 were used to amplify *ctaG* and *surf1c* from genomic DNA purified by the G-spin genomic-DNA-Extraction-Kit (Intron Biotechnology, Inc., Korea). The plasmid carrying *ctaG* was constructed by cutting pFH11 by SacI and introducing the corresponding SacI-digested PCR-product. The plasmid carrying *ctaG* and *surf1c* was constructed in an analogous manner using the respective PCR-products following a restriction digestion. T4 DNA-Ligase (Epicentre Biotechnologies, Madison, Wisconsin, USA) was used for ligation. All modifications were verified by DNA sequencing (Eurofins MWG Operon, Ebersberg, Germany). The expression of *ctaG* was checked via Western-blotting using anti-CtaG-antibodies (kindly provided by Achim Hannappel, Goethe University, Frankfurt, Germany).

Table 1
Sequences of primers used for the construction of different expression plasmids for C*c*O.

Modifications	Primer-sequence 5′ to 3′
rec. WT, rec. WT with chaperones	GATCGATCTAGAAACAGGCGAGTCCGTCGG
rec. WT,	GATTCAGAGCTCTCAATGCGCGTGTGCGCG
ctaG	ATCTAGGAGCTCAGGAGGGGCGAACCAATGAGC
ctaG	ATCGAGCTCATCAGTACTTCAGTTTACGGTCGGTTC
surf1c	TCAGAGCTCATTAGTACTAGGAGGGCCATTTCGATGCGCCGT
surf1c	ATTGAGCTCTAAAGTACTCTAGAATTGCCGCTG

2.2. Triparental mating

Triparental mating was performed using *E. coli* DH5α carrying the target plasmid, the *E. coli* helper strain RP4-4 and *P. denitrificans* AO1 [10] as described elsewhere [20]. The mixing ratio of the strains used was 1:1:3 respectively.

2.3. Cell growth and membrane preparation

P. denitrificans was grown aerobically at 32 °C and 170 rpm overnight using 5 l baffled flasks and 2 l succinate-media [21]. Cells were harvested at 10,500 ×*g* and resuspended in 200 mM K_2HPO_4/KH_2PO_4 (KP_i), pH 8.0, 1 mM ethylenediaminetetraacetic acid (EDTA). Cells were disrupted using a microfluidizer (Microfluidics, Newton, Massachusetts, USA) with a pressure of 1300 bar. Cell debris was removed by a centrifugation step at 6900 ×*g*. Membranes were pelleted at 125,000 ×*g* and resuspended in 50 mM KP_i, pH 8.0, 1 mM EDTA.

2.4. Solubilization and protein purification

Membranes were solubilized with a 1:2 ratio of protein to β-dodecylmaltoside (DDM) (Glycon GmbH, Luckenwalde, Germany) at protein concentrations of 15–25 mg/ml, adding 98.4 mM KP_i, pH 8.0, 1.7 mM EDTA, 90 μg/ml Pefabloc, and 135 μg/ml avidine and stirred on ice for 12 min. After 12 min 32.7% (v/v) *E. coli* periplasmic extract containing a strep-tagged antibody fragment was added [22]. To allow binding of the antibody fragment to C*c*O stirring was continued for additional 12 min followed by a 70 min centrifugation step at 235,000 ×*g*. The supernatant was loaded onto a Strep-Tactin superflow high capacity (IBA GmbH, Goettingen, Germany) affinity chromatography column and unbound material was removed by washing with 50 mM KP_i, pH 8.0, 1 mM EDTA, 0.05% (w/v) DDM. The bound protein was eluted with 50 mM KP_i, pH 8.0, 1 mM EDTA, 0.05% (w/v) DDM, 10 mM desthiobiotin (IBA GmbH, Goettingen, Germany), concentrated using Centriprep concentrators (Millipore, Billerica, MA, USA) and further concentrated using Amicon concentrators (Millipore, Billerica, MA, USA), both with an exclusion volume of 50 kDa. The C*c*O concentration was determined spectrophotometrically using an extinction coefficient of $\varepsilon_{425\ nm} = 158\ mM^{-1}\ cm^{-1}$ [23].

The two-subunit C*c*O was prepared according to the protocol described for the four SU C*c*O but the membranes were solubilized with 3% (v/v) lauryldimethylamine-N-oxide (LDAO) in addition to the 4.5% (w/v) DDM. Affinity chromatography was performed with 0.08% (v/v) of LDAO in all buffers instead of DDM. The eluted fraction was submitted to anion exchange chromatography for detergent exchange. Ten column volumes of 50 mM KP_i, pH 8.0, 1 mM EDTA, 0.05% (w/v) DDM were used to wash the column after binding. The protein was eluted with 600 mM KP_i, pH 8.0, 1 mM EDTA, 0.05% (w/v) DDM. The subsequent steps were the same as for the purification of the four subunits enzyme.

2.5. Measurement of O_2 concentration

Oxygen consumption (oxidase activity) as well as production (catalase activity) was measured using an Ox-MR-Electrode linked to a Picoammeter PA 2000 (Unisense A/S, Aarhus, Denmark) at 20 °C. Signals were recorded using a A/D converter and the Micro Trace Basic software (Unisense A/S, Aarhus, Denmark). During the measurements the samples were stirred in 2 ml glass vessels with a sample volume of 600 μl.

Oxidase activity was measured in 30 mM KP_i, pH 7.0, 0.05% (w/v) DDM, 1 mg/ml phosphatidylcholine, 0.3 mM tetramethylphenylenediamine (TMPD), 3 mM sodium ascorbate and 40 μM cytochrome *c*. C*c*O (final concentration 5 nM) was added to start the reaction.

Catalase activity of C*c*O was monitored in 30 mM KP_i, pH 7.0, 0.05% (w/v) DDM, 1 mg/ml phosphatidylcholine and 50, 75, 125, 200, 250, 450, 500 or 600 μM hydrogen peroxide corresponding to 100 to 1200 equivalents with regard to C*c*O concentration. After addition of hydrogen peroxide, a slight oxygen production was visible due to spontaneous decomposition of H_2O_2. C*c*O (500 nM) was added to start the reaction. After approximately 5 min the buffer was oversaturated with oxygen and started to release oxygen. Only the linear part of the initial slope after addition of C*c*O was used for data analysis (the first 20 to 30 seconds after C*c*O addition). The dependence of the catalase activity of C*c*O on incubation at higher temperatures was measured as described above, samples were heated for 10 min to the indicated temperature, cooled on ice and used for measurements. A mixture of cytoplasm and periplasm was prepared as described in the membrane preparation part, except that the supernatant, containing the periplasm and cytoplasm, was used. Measurements concerning the catalase activity of the mixed cytoplasm and periplasm were heated as described, cooled on ice and measured. Hydrogen peroxide was used in a concentration of 1 mM, whereas a 1:600 dilution of the supernatant of the membrane preparation was used.

The pH dependence of the oxidase and catalase activities was measured in steps of 0.5 pH units using 30 mM citrate (pH 4.5–6.5), 30 mM potassium phosphate (pH 6.5–8.0), 30 mM Tris/phosphoric acid (pH 8.0–9.0) or 30 mM glycine (pH 9.0–10.0) pH-buffers in the presence of 0.05% (w/v) DDM. The pH dependence of the catalase activity was measured with 1 μM C*c*O and the H_2O_2 concentration increased accordingly.

The inhibitory effect of sodium azide (38 mM), potassium cyanide (1 mM), carbon monoxide (CO aerated buffer) and ammonia (20 mM $NH_3/(NH_4)_2SO_4$) on the catalase activity of C*c*O (1 μM for measurements with N_3^-, CN^- and CO and 2 μM for measurements with NH_3) was investigated in 30 mM KP_i, pH 7.0, 0.05% (w/v) DDM and 1 mg/ml phosphatidylcholine. Measurements using CO were performed in the dark in order to avoid flash dissociation of CO. The influence of 100 mM 3-A amino-1,2,4-triazole was analyzed by using a protein concentration of 500 nM C*c*O or 4 units bovine liver catalase (Sigma-Aldrich, Taufkirchen, Germany) respectively. The hydrogen peroxide concentration was 500 μM. Data were analyzed with Origin 7 (Additive GmbH, Friedrichsdorf, Germany).

2.6. Determination of metal content

The metal content of the rec. WT and the ATCC WT C*c*Os was determined by the Ion Beam Center of the University of Surrey using microPixe (micro particle induced x-ray emission) as described elsewhere [24]. This method allows internal calibration based on the sulfur atoms of the protein.

2.7. Capillary differential scanning calorimetry

Thermal stability of both wild type C*c*Os (ATCC and rec.) was determined using a VP-capillary differential scanning calorimeter (GE Healthcare, Buckinghamshire, UK) with a protein concentration of 5 mg/ml in 10 mM KP_i, pH 8.0, 0.2 mM EDTA, 0.01% (w/v) DDM. The sample was scanned with a scanning rate of 90 °C / h in low feedback mode. Data analysis was done with Origin 7 (Additive GmbH, Friedrichsdorf, Germany).

3. Results

In order to compare the ATCC WT C*c*O and the variants in a more stringent way we constructed a recombinant WT (rec. WT) using the same deletion strain AO1 [10] as for our variants (lacking *ctaDI*, *ctaDII*, *ccoN* and *ORF4*). The strain was supplemented with a low copy number plasmid carrying *ctaDII*, which codes for SU I of C*c*O (without any change in the amino acid sequence). The presence of all four protein SU in the rec. WT C*c*O was verified by sodium-dodecylsulfate polyacrylamide gel-electrophoresis (SDS-PAGE). X-ray crystallography as well as mass spectrometry analysis revealed no signs for any covalent modification of SU I (data not shown) besides the crosslink between H276 and Y280 (*P. denitrificans* numbering). Absorbance spectra of the two WT C*c*O showed no significant difference, apart from a very slight broadening towards the blue in case of the rec. WT C*c*O (see Fig. 1). The rec. WT C*c*O showed no difference in oxygen reduction activity but a 20-fold increased catalase activity compared to the ATCC WT C*c*O (see Fig. 4). This very surprising result required further investigation.

3.1. Cloning, expression and metal determination

Incorrect incorporation of the metal centers might be one reason for the observed differences. Since the UV/vis spectra showed no considerable deviations, a lack or deficiency in heme a_3 was ruled out. Therefore, we assumed that Cu_B might be incorporated incompletely or incorrectly leading to structural changes in the active center and, consequently, an increased probability for the heme a_3 dependent catalase reaction in the rec. WT C*c*O.

A potential over-expression of the plasmid encoded SU I might lead to a lack of Cu and/or Cu inserting chaperones. The gene products of *ctaG* and *surf1c* act as chaperones for the co-translational insertion of Cu as well as of heme *a*, respectively [25,26]. To meet the potential requirement for a higher amount of chaperones, we constructed two plasmids carrying either only an additional copy of *ctaG* or a copy of both chaperone genes (*surf1c* and *ctaG*) in addition to the gene encoding SU I. Expression of *ctaG* and *surf1c* was verified by Western-blotting.

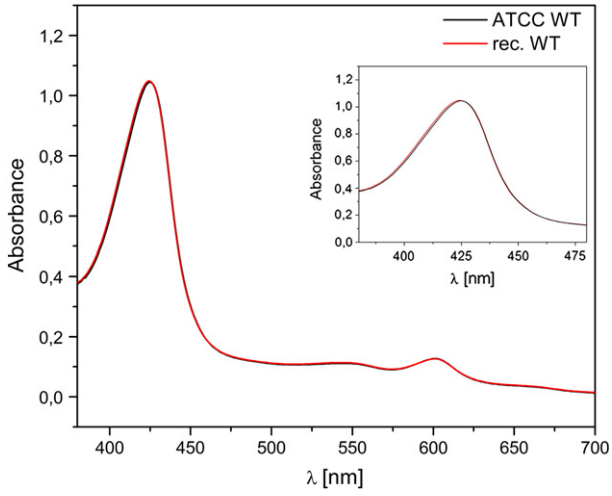

Fig. 1. UV/vis spectra of the purified ATCC WT C*c*O (black) and rec. WT C*c*O (red). A very slight blue shift of the absorbance spectrum obtained from the rec. WT C*c*O is the only spectral feature distinguishing the two preparation. The inset shows a close up in the area from 380 to 480 nm.

The presence of all subunits in the different WT CcOs was verified by SDS-PAGE. The rec. WT CcO and the chaperone added WT CcOs did not show any significant differences in absorbance spectra as compared to the ATCC WT CcO except the very minor broadening towards the blue in the spectrum of the rec. WT CcO (see Fig. 1).

To exclude insufficient Cu_B insertion as the reason for the differences between the rec. WT and the ATCC WT CcOs, the completeness of the insertion of the metal cofactors in the rec. WT CcO was examined using microPixe. No irregularities in iron content were found, therefore heme *a* and heme a_3 were being inserted in all CcOs. All WT CcOs exhibited a copper ratio of three copper atoms per CcO molecule. Since the rec. WT CcO showed no differences to the ATCC WT CcO, the metal content of the rec. WT CcOs isolated from the strains with additional chaperone genes was not determined. Determination of the Mn content did not show any differences between the rec. WT CcO and ATCC WT CcO. The magnesium content of the samples could not be determined due to technical restrictions. Since obvious reasons for the described difference such as subunit and metal composition could be ruled out, we characterized the catalase and oxidase activity in detail.

3.2. Characterization of oxidase and catalase activity

The oxidase activity was determined for all WT CcOs and did not differ significantly from previously reported values (400 to 500 e^-/s) [27]. Measurements using different buffer systems ranging from pH 4.5 to pH 10 resulted in an overall pH optimum of pH 7.0 regarding both, the oxidase and catalase activity (for an example of the pH dependence measurements see Fig. 2).

3.3. Kinetics of the catalase activity of CcO

To characterize the catalase activity of CcO, the Michaelis–Menten constant K_M and the turnover number k_{cat} (which is equal to V_{max} considering a single CcO complex) were determined. The hydrogen peroxide dependent oxygen production at eight different hydrogen peroxide concentrations was measured in a ratio of CcO to hydrogen peroxide of 1:100 to 1:1250 (see Fig. 3c). The influence of hydrogen peroxide concentrations exceeding the described ratios was tested as well but not further considered due to experimental restrictions. The raw data (see Fig. 3a and b for an example of the raw data) was processed and fitted with the Michaelis–Menten equation to yield a Lineweaver–Burk plot with a y-axis intercept of $1/V_{max}$ and a slope of K_M/V_{max} (R-values 0.99–0.92) (see Fig. 3d). k_{cat} was obtained by dividing the oxygen concentration by the protein concentration used. The values of k_{cat} and K_M for the different WT CcOs are summarized in Fig. 4.

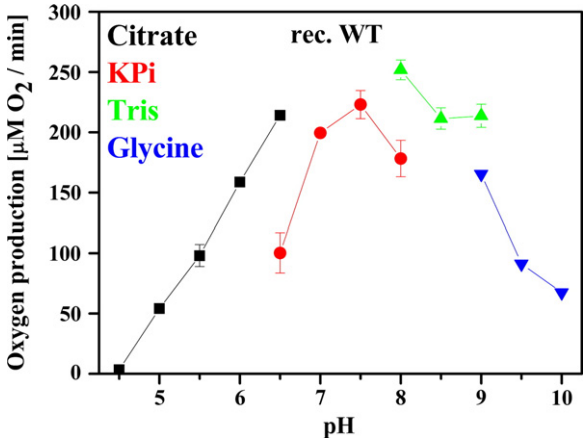

Fig. 2. pH dependence of the catalase activity of the rec. WT. The pH dependence of rec. WT CcO catalase activity (oxygen production) is shown exemplarily. Measurements were done in 30 mM of the respective buffer with 1 µM cytochrome *c* oxidase and 1.2 mM hydrogen peroxide. The measurements show a relatively sharp pH-optimum at pH 7.5. Analysis of the pH dependence of the oxidase and catalase reaction in the ATCC WT CcO and rec. WT CcO yielded an overall pH optimum of pH 7.0.

The rec. WT CcO (k_{cat} = 200 min^{-1} ± 33 min^{-1} corresponding to a second order rate constant of 3274 M^{-1} sec^{-1} ± 456 M^{-1} sec^{-1}) showed a 20-fold increase in k_{cat} compared to the ATCC WT (k_{cat} = 10 min^{-1} ± 2 min^{-1} corresponding to a second order rate constant of 129 M^{-1} sec^{-1} ± 41 M^{-1} sec^{-1}). The K_M-values of the rec. WT CcO did not show significant differences compared to the ATCC WT CcO. Co-expressing the rec. WT with additional chaperones, either CtaG alone or both Surf1c and CtaG, resulted in a decrease of k_{cat} by ~25% and ~50% respectively, without changing the K_M-value significantly (see Fig. 4).

The catalase activity of the rec. WT CcO was checked directly after purification and after freezing to test whether this activity is related to freeze-thaw cycles and caused by partly denatured enzyme. The k_{cat} catalase activity of freshly prepared rec. WT was slightly faster oxidase and catalase activity decreased by ~20% after one freeze-thaw cycle, showing that the catalase activity is not *a priori* caused by freeze-thaw cycles. Nevertheless one could argue that the catalase activity is caused by partly denatured enzyme. Therefore CcO was heated prior to measurements. Heating to 40 °C for 10 min decreased the catalase activity by ~60% and heating to 50 °C for 10 min decreased the catalase activity by 98%. Heating the protein to 60 °C, 70 °C and 80 °C resulted in a complete loss of catalase activity. In contrast to that, the catalase activity of the cytoplasm/periplasm showed a different dependence on incubation at different temperatures. The catalase activity of the cytoplasm/periplasm stored at 4 °C was set as 100% value, similar to the CcO preparation. We found an increase of the cytoplasmic/periplasmic catalase activity from 100% to 134% upon incubation at 40 °C, and a decrease by 97% upon incubation at 50 °C and no catalase activity upon incubation at 60 °C, 70 °C and 80 °C. The results of these control experiments, in particular the different behavior of the cytoplasmic/periplasmic catalase activity and the catalase activity of CcO upon incubation at 40 °C show that the catalase activity of CcO is not caused by a contamination with a cytoplasmic/periplasmic catalase. Additionally, we measured the influence of the broad range catalase inhibitor 3-amino-1,2,4-triazole [28] on the catalase activity of CcO and found no influence up to a concentration of 100 mM, whereas at this concentration and at similar reaction conditions bovine liver catalase was inhibited by approximately 56%. This result adds additional evidence that there is no contaminating catalase present.

To learn more about the intermediates of the binuclear center, especially of heme a_3, involved in the catalase activity of CcO, the reaction was probed using different inhibitors like cyanide, azide, carbon monoxide and ammonia (for a detailed description of the influence of ammonia see [29]). A 99% inhibition of the catalase activity was achieved by using either cyanide (1 mM) or azide (38 mM). To ensure that CO binding was not inhibited by light, the experiments using CO were performed in the dark. The use of CO did not result in any inhibition of the reaction, whereas the use of 20 mM ammonia even resulted in an increase in catalase activity at pH 9. Whenever we performed these experiments at pH 6 an inhibitory or activating effect of ammonia could not be detected. This observation was surprising and we therefore tested the influence of ammonia on oxidase activity, too; oxidase activity in presence of ammonia (20 mM) at pH 9 decreased, whereas the use of ammonia at pH 6 did not cause any inhibition [29].

3.4. Capillary differential scanning calorimetry

Capillary DSC was used to compare the thermal stability of the rec. WT CcO and the ATCC WT CcO. Both four subunit preparations showed at least five different transition temperatures with midpoint temperatures (T_Ms) around 30 °C, 43 °C, 50 °C, 65 °C and 79 °C (see

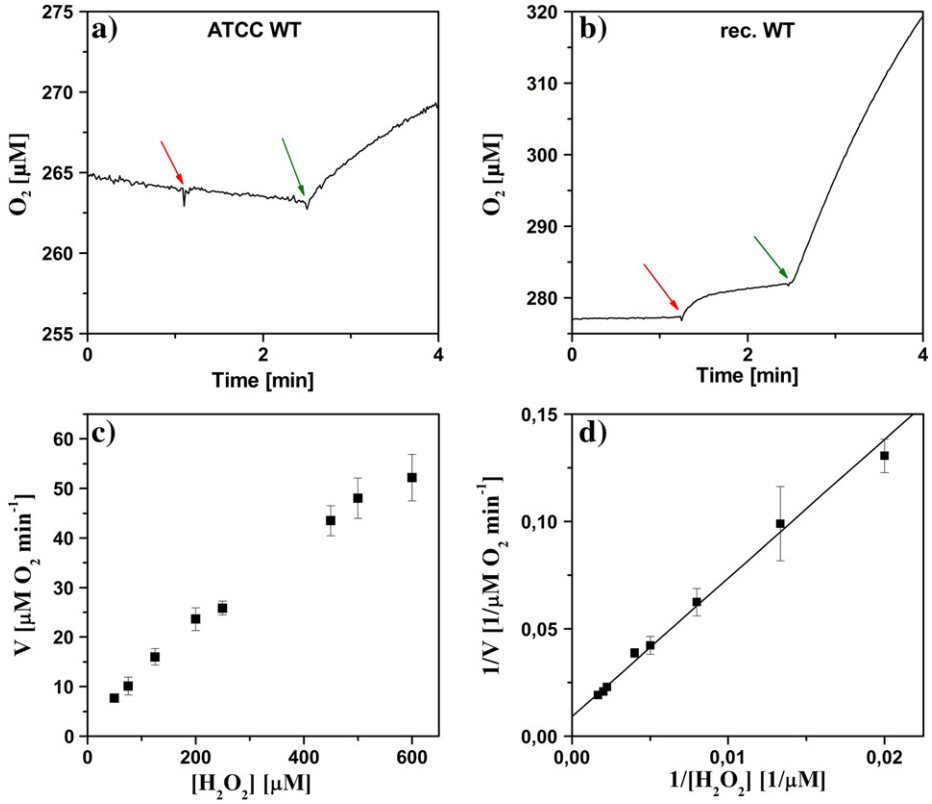

Fig. 3. Raw data of the catalase activity of the ATCC WT C*c*O (a)) and rec. WT C*c*O (b)) at a hydrogen peroxide concentration of 250 µM. The red arrow denotes the addition of hydrogen peroxide and the green arrow the addition of C*c*O. The reaction conditions were 30 mM KP_i, pH 7.0, 0.05% (w/v) DDM, 500 nM C*c*O, 1 mg/ml phosphatidylcholine. Different oxygen concentrations in the buffer system prior to addition of hydrogen peroxide are due to slight temperature differences in the measuring setup. The raw data was further processed via data analysis according to the Michaelis–Menten equation. c) Velocity of oxygen production dependent on hydrogen peroxide concentration of the rec. WT C*c*O. The graphic shows that the hydrogen peroxide concentration is not limiting as evident by the linear shape without any sigmoidal component. Each data point represents the average of four independent measurements. d) shows the Lineweaver–Burk plot of the measurements of c). Linear regression was done to determine K_M and V_{max}. Actual data is displayed in Fig. 4.

Fig. 5a). Measuring in addition denaturation of the core enzyme which consists of SU I and SU II and the Fv-antibody fragment alone, all transition temperatures could be assigned to different events (see Fig. 5c and d). The T_M around 30 °C is caused by a detergent effect visible in all samples with buffers containing DDM. The increase in heat capacity at 43 °C is related to the melting or detachment of SUs III and IV. The T_M at 50 °C by the Fv-antibody fragment, as indicated by its existence in the Fv-antibody control, whereas the peak around 65 °C shows the melting of SU I and II, and the signal at 79 °C is probably related to the detachment of the bound Fv-antibody fragment from SU II.

Both WT C*c*Os show a preparation dependent shift of the transition midpoint temperature of approximately 2 °C of the signal at 65 °C as well as a preparation dependent shift of the signal at 30 °C. We assume that this shift could occur because of different amounts of natural lipids and detergent molecules still attached to the enzyme. Comparing both WT C*c*Os a shift of the T_M value assigned to the melting of SU I and SU II to higher temperatures by approximately 5 °C was observed for the rec. WT C*c*O.

Surprisingly, the rec. WT showed a significant decrease of thermal stability after storage at −80 °C for six days and thawing/freezing the protein three times. The signal at 43 °C related to the melting/detachment of SU III and IV increased and an additional signal with a transition midpoint temperature of 35 °C appeared. The signal at 30 °C, related to detergent effects, shifted to approximately 18 °C with a shoulder appearing at 22 °C after six days of storage at −80 °C. These effects were not observed for the ATCC WT C*c*O.

4. Discussion

4.1. Differences between recombinant and true forms of C*c*O

In this study we demonstrate that the encoding of one protein subunit of C*c*O on a low copy number plasmid leads to a significant difference in a catalytic side reaction of C*c*O, namely a twenty-fold increase of k_{cat} of the catalase activity of the rec. WT C*c*O compared to the ATCC WT C*c*O. The deletion of the chromosomally encoded gene *ctaDII* (coding for SU I present in aa_3 C*c*O), is complemented on a low copy number plasmid controlled by the promoter of the *cta*-operon. However, the turnover of bovine catalase is by 8 orders of magnitude higher than that of C*c*O, showing that the catalase activity of C*c*O is clearly a side reaction [30].

It was shown that differences between recombinant proteins and proteins isolated from native sources may exist [31,32,7]. Orlova et al. described a significantly altered accessibility of certain amino acids on the surface of a recombinant protein compared to the non-recombinant one. This result shows that structural differences between recombinant and non-recombinant proteins are possible [32]. Kenakin described changes in the reaction of G-protein coupled receptors with agonists when the receptor was produced by over-expression of its gene / cDNA [31]. Butenas et al. reported changes in the specific activity of a protein when the protein was obtained in a recombinant form [7]. These differences were partly caused by different post-translational modifications in a different organism chosen for the production of the recombinant protein (heterologous expression). Besides different

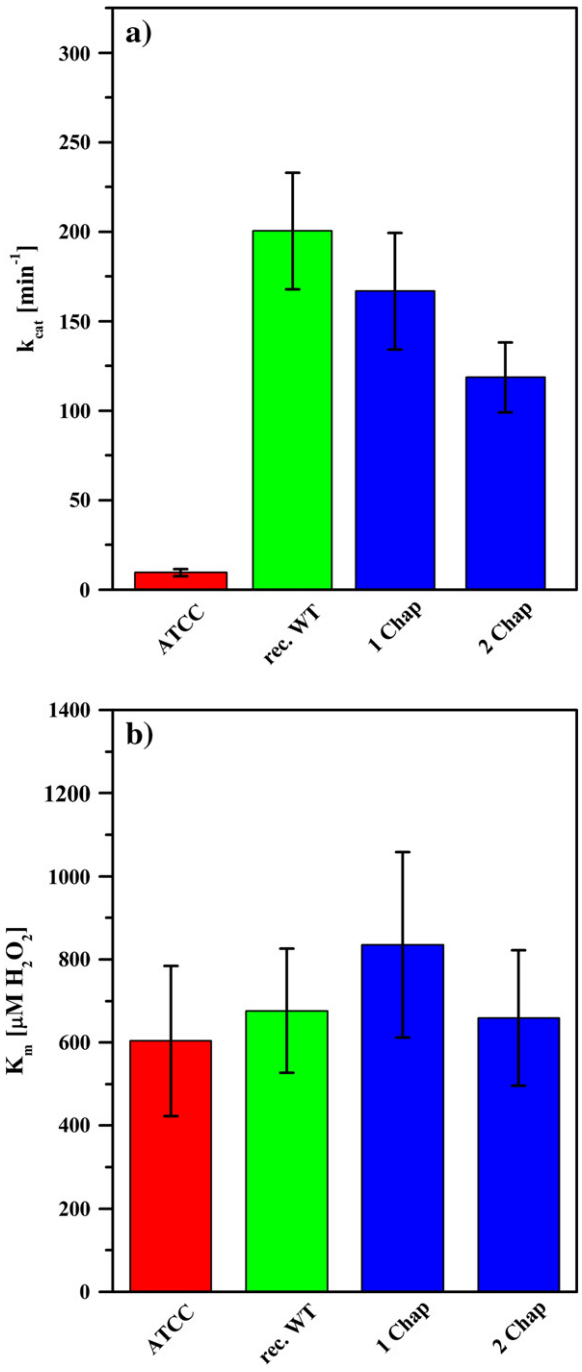

Fig. 4. Kinetic parameters of the catalase activity of WT CcOs. a) k_{cat} values of all WT CcOs are shown as determined according to the Michaelis–Menten equation. The ATCC WT CcO (red) shows a k_{cat} of 9.6 $min^{-1} \pm 2$ min^{-1} (corresponding to a second order rate constant of 129 $M^{-1}sec^{-1} \pm 41$ $M^{-1}sec^{-1}$), whereas the rec. WT CcO (green) possesses a k_{cat} of 200.4 $min^{-1} \pm 32.6$ min^{-1} (corresponding to a second order rate constant of 3274 $M^{-1}sec^{-1} \pm 456$ $M^{-1}sec^{-1}$). A significant decrease of k_{cat} compared to the rec. WT CcO was observed for the rec. WT CcOs supplemented with chaperones (blue, 1 Chap: additional copy of *ctaG*, 2 Chap: additional copy of *ctaG* and *surf1c*). $n = 8$. b) Determination of K_M-values using the Michaelis–Menten equation. Differences in K_M values for the different WT CcOs are not significant.

expression levels of recombinant proteins compared to other proteins in the cell, also differences in conformation / structure of recombinant proteins have been observed. The rec. WT CcO is homologously produced in *P. denitrificans*, therefore codon usage, posttranslational modifications as well as membrane related transport mechanisms are unaffected. Metal determinations did not show any significant differences in copper, iron and manganese content, ruling out any influence of manganese ions on the catalase activity of CcO. In addition, CcO grown in a manganese-free medium still shows the catalase activity. If the catalase activity would be due to free heavy metal ions, heating the solution should not change the catalase activity at all. In contrast to that, we found a nearly complete loss of the catalase activity upon heating for 10 min to 50 °C. These results exclude that contaminating free heavy metal ions are responsible for the catalase activity of CcO. The decrease of the catalase and oxidase activities after the first freeze-thaw cycle also shows that the catalase activity is a specific side reaction of CcO and not a reaction related to partly denatured protein.

One may argue that the catalase activity is due to a contaminating catalase present in the preparation. If the amount of catalase needed to reach the catalase activity of the rec. WT CcO is calculated (assuming a turnover number of 40,000,000 s^{-1} for the catalase), the amount of contaminating catalase in the sample would be in the low femtomole region. All wild type CcOs presented in this work exhibit different and well reproducible k_{cat} values despite identical preparation and growth conditions. The same can also be shown for at least four different variants, exhibiting different k_{cat} values for the catalase activity (manuscript in preparation) despite identical growth and preparation conditions. The k_{cat} values of different preparations of the wild type CcOs (two independent preparations of the identical protein in terms of ATCC WT CcO i.e. from different cultures, see Fig. 4a) and variants are highly reproducible. Therefore, the amounts of contaminating catalase had to be systematically different between the wild type CcOs despite identical growth and preparation conditions and also between the variants, but similar in the various preparations of the same CcO. Since this is very improbable, we can exclude that a contaminating catalase causes the observed differences in the catalase activities of the various CcOs. The different behavior of the cytoplasmic/periplasmic catalase activity and the catalase activity of CcO upon incubation at 40 °C supports that the catalase activity of CcO is not caused by a contaminating cytoplasmic/periplasmic catalase. In addition, measurements performed in the presence of the catalase inhibitor 3-amino-1,2,4-triazole showed no effect on the catalase activity of CcO, providing additional evidence that a contaminating catalase is not the reason for the described results.

The potential overproduction of SU I, although a low copy number plasmid was used, may have caused a spontaneous not chaperone-assisted and therefore not fully correct insertion of the metal cofactors. Slight modifications in the overall structure leading to variations in the interactions of amino acids with each other and with solvent molecules might induce the increase in catalase activity. Hence, supplementing the plasmid of the rec. WT with an additional plasmid-borne copy of the genes for Cu and heme *a* inserting chaperones *ctaG* alone or *ctaG* and *surf1c* resulted in a significant decrease of k_{cat} but was not sufficient to reach the ATCC WT level. The reason for the observed difference is therefore clearly based at the structural level. The slight broadening of the absorbance of the rec WT CcO may be an indication that the environment of the heme groups might slightly differ from that of the ATCC WT CcO although such differences have not been seen by X-ray crystallography. It might also be possible to reduce the catalase activity of the rec. WT even further by adding generally available chaperones of *P. denitrificans* but this has to be tested.

A comparison of the crystal structures of the two-subunit ATCC WT CcO and the two-subunit rec. WT CcO at a resolution of 2.7 Å [33] and 2.25 Å [34], respectively, revealed no noticeable difference in structure. This resolution might be too low to detect very minor differences, and, in addition, the structure of the rec. WT was determined by molecular replacement using the structure of the ATCC WT CcO leading to model bias. We cannot rule out that there are differences between the structures of the four-subunit enzymes.

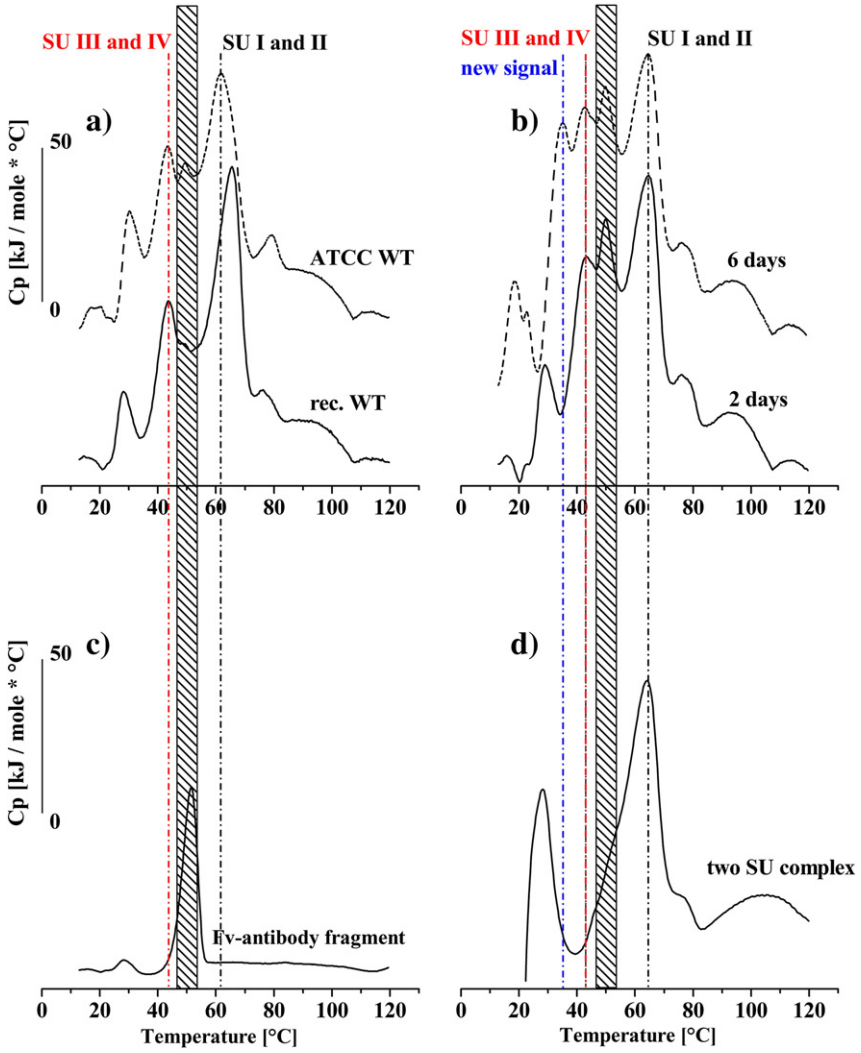

Fig. 5. Differential scanning calorimetry measurements of the ATCC and rec. WT CcOs with different subunit composition and the Fv-antibody fragment without CcO. Different intensities of the transition temperature at 50 °C are due to different amounts of free Fv-antibody fragment in the different preparations. Freeze–thaw cycles lead to the detachment of the Fv-antibody fragment from SU II. The gray shaded square covers the signal arising from the Fv-antibody fragment. The red line marks the signal resulting from the denaturation / detachment of SU III and IV and the black line the corresponding signal of SU I and II. a) Comparison of the four-SU ATCC WT CcO (solid line) and the four-SU rec. WT CcO (dashed line) (stored at −80 °C for two days). The transition temperature around 61 °C shifts to approximately 64 °C for the rec. WT CcO. b) Evolution of the transition temperatures of the rec. WT CcO, the solid line shows the four-SU rec WT CcO after 2 days storage at −80 °C and two freeze/thaw cycles. The dashed line shows the same protein after six days of storage and three freeze/thaw cycles. The blue line marks the new signal appearing in the rec. WT upon aging. c) DSC-data of the Fv-antibody-fragment without CcO, showing a distinct signal with a transition midpoint temperature of 50 °C. d) Typical measurement of a two-SU protein complex of the rec. WT CcO. The signal with a transition midpoint temperature of about 43 °C is missing compared to the four-subunit form of the protein and is therefore assigned to the release/denaturation of subunits III and IV.

However, the accuracy of the structure of the four-subunit ATCC WT CcO is considerably lower and there is no structural data on the four subunit rec. WT CcO.

Another possible reason for the difference of the catalase activity of the rec. WT CcO and the ATCC WT CcO might be that CcO may contain an essential lipid species suppressing its catalase activity. This lipid species may not be present in sufficient amounts in the recombinant system. In this context it is interesting to note that phosphatidylglycerol containing vaccenate as fatty acid side chains has been found in the CcO from *P. denitrificans* [35]. A lack of this lipid species could cause a higher accessibility to the active site for hydrogen peroxide.

Ji et al. [36] showed that the CcO is able to adopt two different conformations, called α and β. They constructed several variants exhibiting different amounts of α and β conformations and found that these two conformers show different oxidase activities and possess different distances between heme a_3 and Cu_B. They also showed that a mutation, which is not directly located near the active center, leads to a decrease in oxidase activity by nearly 40%. The influence of such distant mutations on the structure of the active center and the enzymatic activity of CcO reveals the sensitivity of the active site and of the catalytic activity of CcO. An increase in catalase activity caused by small changes in the environment of the binuclear site seems therefore feasible.

4.2. Capillary Differential Scanning Calorimetry

We compared both wild type CcOs using capillary DSC and showed that both enzymes exhibit no differences in thermal stability directly after purification. Surprisingly, the rec. WT CcO showed an altered behavior after storage at −80 °C for 6 days and thawing/freezing the protein three times. The new transition temperature at 35 °C which appears after 6 days could be due to detached and/or denatured subunits III and IV. An interaction of the detached subunits III and IV with DDM micelles could result in an altered signal, shifting the DDM-signal from 30 °C to

18 °C with a shoulder at 22 °C. Since the ATCC WT CcO did not show any of these features upon storing the enzyme or thawing/freezing we conclude that the rec. WT CcO is more labile than the ATCC WT CcO. These results show clearly that there are time dependent changes in structural features in the rec. WT CcO that are not present in the ATCC WT CcO.

The difference in the time dependence of the thermal stability between the two wild type CcOs seems to be puzzling at first. In addition to the existence of the α- and β-forms of the enzyme [36], Elferink et al. [37] observed the appearance of a second conformation of cytochrome *c* oxidase after storing the enzyme at room temperature. Morin and Freire showed in 1991 [38] that enhanced ionic strength decreases the stability of yeast cytochrome *c* oxidase and speculated that an increased ionic strength favors the formation of a second conformation of CcO. This second conformation shows a decreased interaction of subunits I and II. A second conformation could also have an altered lipid content. All experiments described show that CcO can exist in various conformational states.

The rec. WT CcO was obtained using a low copy number plasmid resulting in a potential over-production of SU I compared to the other subunits. That the plasmid encoded SU I actually is overproduced has not been directly shown so far. It has not been possible to determine mRNA levels because it is very difficult to prepare good-quality mRNA of *P. denitrificans* and multiple trials (Ludwig and coworkers, unpublished) did not yield any positive results. The next possibility is to check for a potential over-production at the protein level. Since only little is known about the assembly of CcO and only one out of the four SUs is plasmid-encoded, this SU might be produced in excess but not assembled into fully functional CcO. The surplus of the plasmid encoded SU I would therefore be degraded. The limiting factor for the biosynthesis of CcO would be the availability of the chromosomally encoded SUs. The amount of CcO made, finally would remain the same, if the production of the chromosomally encoded SUs is not upregulated. We considered testing the possibility of an over-production of SU I but found it very difficult because of technical restrictions. Nevertheless, the presented results, in particular the decrease of k_{cat} for the catalase reaction of the rec. WT upon addition of chaperones supports the hypothesis of a potential over-production of the plasmid encoded SU I.

We hypothesize that there might be a lack of a chaperones, preventing formation of 1:1 complexes between all SU I molecules and the chaperones. The chaperone-free and chaperone-bound forms of SU I might adopt different conformations leading to differences in metal co-factor insertion and formation of different water networks. These structural differences and the altered interaction of SU I and II might result in a faster relaxation of the rec. WT into a second conformation, similar to the ones described in the preceding paragraph but not necessarily the same [38]. The different interactions of SU I and II in the second conformation could lead to a faster detachment of subunits III and IV as suggested by the capillary DSC experiments. The rec. WT CcO may relax faster than the ATCC WT CcO into a second conformation of the oxidized form due to small structural defects caused by plasmid coding of subunit I. In this second conformation the CcO may exhibit an increased catalase activity e.g. due to an increased accessibility of the active center for hydrogen peroxide. This could also be the result of a different lipid species present in the ATCC WT CcO decreasing the accessibility of the active center for hydrogen peroxide.

4.3. The catalase activity of CcO

We studied the decomposition of hydrogen peroxide by CcO by direct detection of the molecular oxygen formed, using a selective micro-redox electrode. We could not find a clear saturation behavior of the oxygen production in the described concentration range. However, very high hydrogen peroxide concentrations led to a saturation like behavior. Therefore, we determined the k_{cat} and K_M values as well as second order rate constants for the described concentration range. Combining the results from catalase and oxidase activity measurements, we found an overall pH-optimum of pH 7.0. Considering the buffer conditions used for the measurements described in this work, our results are in agreement with previous data on the pH optimum of the oxidase activity of bovine CcO [39]. The catalase activity of CcO was originally discovered by Orii and Okunuki in 1963 [12], who observed changes in the UV/vis spectrum of CcO due to the reaction with hydrogen peroxide and determined the decreasing H_2O_2 concentration by titration with potassium permanganate. Konstantinov and co-workers [40-42] further investigated the reaction of CcO with H_2O_2 by correlating the UV/vis spectral changes with the hydrogen peroxide derived artificial **P** and **F** intermediates. It has been postulated that the **P** intermediate is converted to the **F** intermediate by accepting an electron from hydrogen peroxide, and the **F** intermediate further to the oxidized form by accepting another electron from hydrogen peroxide [43]. Two superoxide radicals would be formed during such a reaction cycle, called pseudocatalase reaction [41].

The decomposition of hydrogen peroxide by the bovine and *Rhodobacter sphaeroides* CcOs e.g. the decrease in H_2O_2 concentration was also studied very recently by Bolshakov et al. using a redox electrode selective for hydrogen peroxide yielding a second order rate constant of ~$2*10^2$ M^{-1} sec^{-1} for the bovine enzyme and of up to 3000 M^{-1} sec^{-1} for the *Rhodobacter sphaeroides* enzyme [13]. The second order rate constants determined for our *P. denitrificans* rec. WT CcO are in good agreement with the data presented in ref. [13] for rec. WT CcO from *R. sphaeroides*. The ATCC WT CcO shows a second order rate constant, which is very close to the second order rate constant determined for the bovine CcO. However, both recombinant CcOs (*R. sphaeroides* and *P. denitrificans*) exhibit a second order rate constant in the same order of magnitude but compared to the bovine enzyme, the second order rate constant is higher by a factor of at least 10, depending on the origin of the CcO. Bolshakov et al. already reported that a second order rate constant of this order of magnitude does not fit the binding rate proposed by Pecoraro et al. [44,13]. Our measurements show that neither a poly-histidine tag nor a bound manganese ion is the reason for this discrepancy. However, the binding rate was determined using spectroscopic techniques, based on the formation of the **P**-intermediate. We found that the optical spectra are independent from the catalase reaction itself (unpublished data) and thus, the catalase activity is not necessarily linked to the binding rate determined by optical spectroscopy. The **F**-intermediate differs from the **P**-intermediate probably only in the Cu_B ligand. Since Cu_B is dispensable for the catalase activity (unpublished data) the formation of the **F**-intermediate and the optical spectra cannot be correlated with the catalase activity. This result makes a correlation of the spectroscopically determined binding rate with the second order rate constant of the catalase activity very difficult. Additionally, it may be possible that the initial reaction of CcO with H_2O_2 leads to a more open conformation in the rec. WT CcO, resulting in an increase in binding rate and in an increase of the second order rate constant.

The enzymatic catalase activity is generally dependent on the availability of an accessible, oxidized heme moiety. Another heme protein showing catalase activity is the oxidized form of hemoglobin, methemoglobin [45,46]. So it is not surprising that CcO with its free sixth binding site of Fe^{3+} in the heme a_3 moiety shows catalase activity.

The catalase activity of CcO with a k_{cat} of 200 $min^{-1} \pm 33$ min^{-1} or 10 $min^{-1} \pm 2$ min^{-1} for the rec. WT CcO and ATCC WT CcO, respectively, is very low compared to true catalases with a k_{cat} of 40,000,000 s^{-1} and can therefore clearly considered to be a side reaction. Evidently the catalase reaction of CcO takes place at the heme a_3 moiety. This statement is supported by the observed complete inhibition of the catalase activity by the heme-ligands cyanide and azide. The use of CO had no effect on the catalase reaction, resulting in the conclusion that a reduced heme a_3 is not involved. The use of ammonia, which appears to bind to Cu_B [29] resulted in an accelerated catalase reaction at pH 9 and a decreased oxidase activity. Ammonia might promote an orientation and polarization of hydrogen peroxide bound to heme a_3, which promotes the dismutation of H_2O_2. Orii and Okunuki showed already in 1963 [12] that the catalase

activity is increased by addition of hydrazine, which probably coordinates to Cu_B in the same manner as ammonia. The influence of manganese, cobalt and zinc on the difference between the ATCC WT C*c*O and the rec. WT C*c*O was ruled out since metal determination revealed either equivalent or lower levels of these metals in the rec. WT C*c*O compared to the ATCC WT C*c*O.

Since the catalase reaction mechanism of C*c*O is not completely understood, a comparison of the catalase reaction mechanism of C*c*O and that of genuine catalases would be useful, requiring further detailed studies.

5. Conclusion

The catalase reaction of C*c*O from *P. denitrificans* was shown to be a true protein related side reaction. Recombinant C*c*O produced in a homologous system shows a 20 fold increase in k_{cat} of the catalase activity. Upon complementing this rec. WT with additional chaperones, the k_{cat} decreases by ~50% The use of a recombinant system, even if homologous, might lead to severe differences between a recombinant wild type protein and a true wild type protein. This fact has to be taken into account when addressing partial reactions or side reactions of C*c*O that go beyond the overall oxygen reducing capabilities of the enzyme. This result has to be kept in mind when planning experiments, comparing recombinant wild type enzymes and true wild type enzymes to each other as well.

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