Research Article

Recombinantly produced cellobiose dehydrogenase from *Corynascus thermophilus* for glucose biosensors and biofuel cells

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Cellobiose dehydrogenase (CDH) is an emerging enzyme in the field of bioelectrocatalysis. Due to its flexible cytochrome domain, which acts as a built-in redox mediator, CDH is capable of direct electron transfer (DET) to electrode surfaces. This rare property is employed in mediatorless "third generation" biosensors. The ability of *Corynascus thermophilus* CDH to oxidize glucose under physiological conditions makes it a promising candidate for miniaturized glucose biosensors or glucose powered biofuel cell anodes. We report for the first time the electrochemical application and characterization of a recombinantly produced CDH in a glucose biosensor. Recombinant CDH from *C. thermophilus* (*rCt*CDH) was expressed by the methylotrophic yeast *Pichia pastoris* (376 U L^{-1}, 132 mg L^{-1}). A comparative characterization of *rCt*CDH and *Ct*CDH shows identical pH optima, K_M values and heme *b* midpoint potentials. In contrast, the specific activity of *rCt*CDH (2.84 U mg^{-1}) and consequently the turnover numbers were ~five-times lower than for *Ct*CDH, which was caused by a sub-stoichiometric occupation of catalytic sites with flavin-adenin-dinukleotid (FAD). The performance of *rCt*CDH-modified electrodes demonstrates the suitability for electrochemical studies. This opens the possibility to engineer the substrate specificity of *C. thermophilus* CDH for specific carbohydrates by rational engineering or directed evolution.

Received	31 JAN 2012
Revised	05 JUN 2012
Accepted	17 JUL 2012
Accepted article online	20 JUL 2012

Supporting information available online

Keywords: Cyclic voltammetry · Enzymes · Glucose · *Pichia pastoris* · Steady-state kinetics

1 Introduction

The extracellular fungal flavocytochrome cellobiose dehydrogenase (CDH, EC 1.1.99.18) is secreted by several wood degrading, phytopathogenic or saprotrophic fungi within the phyla of Basidiomycota and Ascomycota. According to their origin, CDHs show different catalytic properties. Basidiomycetous class-I CDHs show a high preference for cellobiose, cello-oligosaccharides and the structurally similar lactose, while conversion of monosaccharides such as glucose and galactose is strongly discriminated [1–3]. In contrast, ascomyceteous CDHs convert glucose at higher turnover rates of up to ~30 s^{-1} [4, 5]. The catalytic turnover of carbohydrates occurs in the flavodehydrogenase do-

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Abbreviations: **CDH**, cellobiose dehydrogenase; **CYT_{CDH}**, cytochrome domain of CDH; ***Ct*CDH**, CDH from *Corynascus thermopilus*; **cyt *c***, cytochrome *c*; **DCIP**, 2,6-dichloroindophenol; **DET**, direct electron transfer**;** **DH_{CDH}**, dehydrogenase domain of CDH; **FAD**, flavin-adenin-dinucleotid; **GDH**, glucose dehydrogenase**;** **GOx**, glucose oxidase; **IET**, intramolecular electron transfer; **NHE**, normal hydrogen electrode; ***rCt*CDH**, recombinantly expressed CDH from *Corynascus thermophilus*

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main (DH_{CDH}), which features the flavin-adenin-dinucleotid (FAD) as cofactor. After the catalytic step, electrons are transferred from the FAD moiety to a surface-exposed heme *b* of the smaller cytochrome domain (CYT_{CDH}), which is connected to DH_{CDH} by a flexible linker. In addition to soluble redox mediators, certain electrode surfaces can act as terminal electron acceptors of CYT_{CDH}. This direct electron transfer (DET) mechanism makes CDH highly interesting for bioelectrochemical applications. The efficiency of CDH modified bioelectrodes depends on DET, but also on the intramolecular electron transfer (IET) between DH_{CDH} and CYT_{CDH} [6]. For basidiomycetous CDHs, the IET – but not the catalytic step at the FAD – is limited to acidic pH, but for some ascomycetous CDHs an IET at neutral/alkaline pH was found [4, 7]. The combination of an efficient IET and DET reaction at pH 7.4 and the ability to oxidize glucose makes CDH from *C. thermophilus* a promising candidate for mediatorless glucose biosensors and biofuel cell anodes to be used under physiological conditions in body tissues and fluids [8–10].

Current glucose biosensors are based on glucose oxidase (GOx) or glucose dehydrogenase (GDH). These enzymes are commercially available and exert a high specific activity, selectivity for glucose and stability. Nevertheless, they have some major drawbacks. Due to their architecture, direct communication with electrodes is not possible. Therefore, they depend on electron shuttling species to contact the electrode. Electrons can be transferred by migrating reaction products (e.g. hydrogen peroxide, "first generation" biosensors) or the help of redox mediators ("second generation" biosensors). First generation glucose biosensors based on GOx are dependent on a stable oxygen concentration for accurate measurements. Second generation glucose biosensors can be affected by varying oxygen concentrations or hydrogen peroxide production. CDH, such as GDH, cannot use oxygen as alternative electron acceptor and circumvent this negative effect [11]. The high operational potential of GOx- and GDH-based biosensors leads to nonspecific oxidation of interfering compounds for instance acetaminophen, an active agent of common painkillers. CDH-based biosensors avoid these negative effects by working at a low operational potential [11]. The much lower K_M values of GOx or GDH for glucose seem to be an advantage compared to CDH. However, experiments showed that the linear range (0.1–30 mM) of a *Ct*CDH glucose biosensor covers the concentration range relevant for the direct determination of the blood glucose level [10].

The production of *Ct*CDH by the fungus is difficult and demands a complex and expensive culture medium [4]. The applicability of a heterologously expressed CDH would circumvent these drawbacks and, even more important, would allow to customize CDH for bioelectronic applications by genetic engineering. Recombinant production of CDH by bacterial expression systems seems to work only for DH_{CDH}, but not for the holoenzyme [12]. Therefore, high reported expression levels and an easy genetic manipulation were the reason to select *Pichia pastoris* as expression host in this work. We report the efficient recombinant expression of *rCt*CDH in *P. pastoris*, its purification and a comparative characterization with fungal *Ct*CDH in respect to physical, catalytic and bioelectrochemical properties.

2 Materials and methods

2.1 Chemicals, strains and vectors

Chemicals for enzyme assays, buffers and media were purchased from Sigma-Aldrich (Steinheim, Germany) and were of the highest purity available. Restriction enzymes, dNTP mix and T4 DNA ligase were from Fermentas (Vilnius, Lithuania) and the Phusion polymerase from New England Biolabs (Ipswich, UK). Synthetic oligonucleotides were synthesized by VBC-Biotech (Vienna, Austria). *Pichia pastoris* strain X-33 and expression vector pPICZα B used for heterologous protein expression are components of the EasySelect Pichia Expression Kit (Invitrogen, CA, USA). CDH from *Corynascus thermophilus* CBS 405.69 (CtCDH) was produced according to a published procedure [4].

2.2 Recombinant enzyme production

The published plasmid pCT1 was used as template for the amplification of *Ct*CDH encoding cDNA (HQ116814.1, [4]) using the primers 5CT-*Bst*BI 5'-ATATTCGAAATGAAGC TTCTCAGCCGCG-3' and 3CT-*Xba*I 5'-ATATCTAGACTA ATACCGCAGGGACAGG-3'. The PCR amplicon was digested with *Bst*BI and *Xba*I and cloned into the equally treated vector pPICZa B. The resulting plasmid pPIC*ctcdh* was linearized with *Sac*I and transformed into electrocompetent *P. pastoris* X-33 cells. Transformants were selected on yeast peptone dextrose (YPD) zeocin plates (100 mg L^{-1} zeocin). The integration of the gene was verified by colony PCR. A 96-deep well plate based screening for a high producing *P. pastoris* transformant was done according to Weis et al. [13] with minor modifications of the culture conditions. Cells were grown at 25°C, 385 rpm and 60% relative humidity for 130 h. The enzymatic activity in the supernatant of each well was measured using the 2,6-dichloroindophenol (DCIP) assay. The best producing clone was selected for production of r*Ct*CDH in a 7-L fermenter (MBR, Wetzikon, Switzerland). The fermentation was run according to Sygmund et al. [14].

2.3 Protein purification

The enzyme was purified using a hydrophobic interaction chromatography followed by an anion exchange chromatography. The purity of the enzyme preparation was

verified by SDS-PAGE. A detailed description of the purification procedure is provided in the Supporting information. *Ct*CDH was prepared as published previously [4]. Homogeneous CDH solutions were sterile filtered, aliquoted and stored at –80°C.

2.4 Molecular properties

Homogenous CDH samples were treated with PNG-ase F (NEB) under denaturing conditions according to the manufacturer's instructions to estimate the amount of glycosylation. The spectra of homogeneously purified *rCt*CDH and *Ct*CDH were recorded at room temperature from 250 to 550 nm in both the oxidized and reduced state using a U-3000 Hitachi spectrometer (Tokyo, Japan). CDH was diluted in 50 mM citrate buffer, pH 5.5 to an absorbance of ~1 at 280 nm and the spectrum was recorded before and shortly after the addition of lactose to the cuvette. The oxidized spectrum was used for determining the purity represented by the ratio of A_{420} to A_{280}. Reconstitution of the recombinant CDH with FAD was done by incubation of the enzyme in citrate-phosphate buffer (50 mM, pH 4.0) containing a 10-fold excess of FAD (100 µM) for 24 h at room temperature. Residual FAD was removed by repeated diafiltration (Ultra-0.5; 10-kDa cut-off; Millipore, MA, USA). The percentage of reconstitution was determined by measuring the DCIP activity before and after the experiment.

2.5 Enzyme assays and kinetic measurements

CDH activity was specifically determined by following the reduction of 20 µM cytochrome *c* (cyt *c*, ε_{550} = 19.6 M^{-1} cm^{-1}) in 100 mM McIlvaine (citrate-phosphate) buffer, pH 7.5, containing 30 mM lactose [15]. The DCIP assay was performed by measuring the time-dependent reduction of 300 µM DCIP at 520 nm in 100 mM McIlvaine buffer at pH 5.5. Initial rates for the determination of pH profiles with DCIP and cyt *c* were determined at 30°C in McIlvaine's buffer ranging from pH 3.5 to 8. Catalytic constants for various electron donors were determined with cyt *c* at pH 7.5. Catalytic constants were calculated using nonlinear least squares regression by fitting the observed data to the Michaelis-Menten equation (Sigma Plot 11, Systat Software, Chicago, IL, USA).

2.6 Voltammetric measurements

Cyclic voltammetry (scan rate 10 mV s^{-1}) and square wave voltammetry were performed at room temperature (20°C) using a Gamry Reference 600 potentiostat (Gamry Instruments, Warminster, PA, USA). Square wave voltammograms were recorded at a frequency of 1 Hz, a step potential of 2 mV and amplitude of 20 mV. A standard three-electrode configuration was used with an Ag|AgCl (3 M KCl) reference electrode (Gamry Instruments) and platinum wire as counter electrode. The buffer, used as electrolytes, was carefully degassed under vacuum and purged with argon prior to experiments. To maintain the inert atmosphere argon was blown over the solution during the measurements. Preparation of thioglycerol-modified disc Au electrodes (BASi, West Lafayette, IN, USA, diameter = 1.6 mm, geometric surface area = 2.01 mm^2) for cyclic voltammetry and square wave voltammetry is described elsewhere [6].

2.7 Amperometric measurements

A three-electrode flow through amperometric wall jet cell was used (BASi LCEC flow cell, radial flow, West Lafayette, IN, USA) containing a custom-made working electrode (graphite electrode modified with CDH), a reference electrode (Ag|AgCl, 3 M NaCl, RE-6, BASi) and a counter electrode block (BASi). The cell was connected to a potentiostat (µSTAT 200, DropSens, Oviedo, Spain). The enzyme modified electrode was press fitted into a Teflon holder and screwed onto the counter electrode block, which kept a constant distance (~1 mm) from the inlet nozzle. The system was controlled by the DropView program (DropSens). The electrochemical cell was connected on-line to a single line flow injection system, in which the carrier flow was maintained at a constant flow rate of 0.5 mL min^{-1} by a peristaltic pump (Minipuls 2, Gilson, Middleton, WI, USA). The injector was a mechanically controlled six-port valve (Rheodyne, Cotati, CA, USA) with a 20-µL injection loop. CDH was immobilized through simple physicochemical adsorption onto the surface of solid spectroscopic graphite electrodes (FP-254, diameter = 3.05 mm, geometric surface area = 7.06 mm^2, Schunk Materials, Heuchelheim, Germany). The electrode was cut, polished on wet emery paper (EasyCUT, P600), sonicated for 10 min, carefully rinsed with HQ water and dried. A 5-µL aliquot of enzyme solution (5 mg mL^{-1}) was spread across the active surface of the electrode. The enzyme electrode was dried at room temperature and stored overnight at 4°C. Before use, the electrode was thoroughly rinsed with HQ water in order to remove any weakly adsorbed enzyme and plugged into the wall jet cell already containing buffer. Prior to substrate injection (50 mM glucose), the electrode was polarized with an applied potential of +200 mV until a stable background current was achieved. The dispersion factor of the flow system was equal to 1.96 according to the Ruzicka and Hansen relationship [16].

3 Results

3.1 Production and purification of recombinant *Ct*CDH

The *P. pastoris* expression plasmid pPIC*ctcdh* was constructed by cloning the nucleotide sequence including

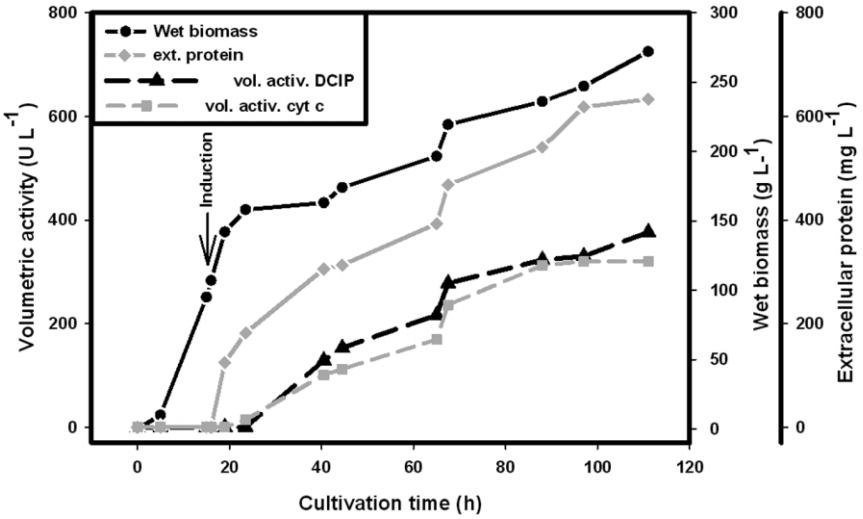

Figure 1. Cultivation of *rCt*CDH in a 7-L fermenter. Left axis: enzymatic activity with DCIP (black triangles) and with cyt *c* (gray squares); right axis: wet biomass (black circles) and extracellular protein (gray diamonds).

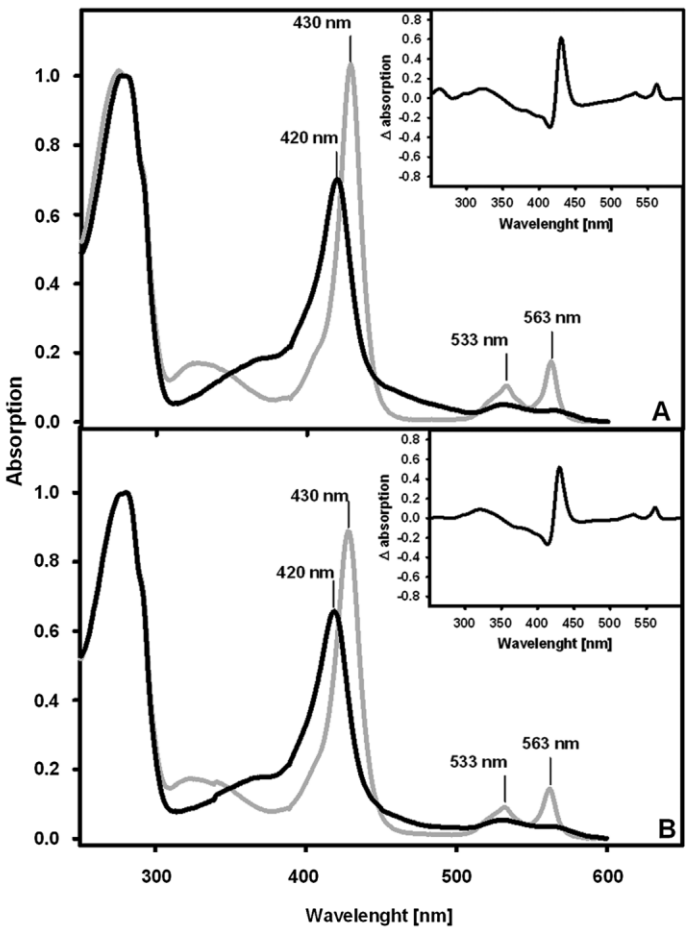

Figure 2. Spectral comparison of *Ct*CDH (**A**) and *rCt*CDH (**B**) in oxidized (black line) and reduced (by a 100-fold molar excess of lactose, gray line). Upper right insets: differential spectra (red-ox).

the native signal sequence into the pPICZα B expression vector under control of the methanol-inducible AOX promoter. A high producing clone (pPIC-*Ct*CDH11) was selected during a 96-deep well plate screening. Production of the enzyme was carried out in a 7-L stirred and aerated bioreactor (Fig. 1). Volumetric CDH activity in the culture supernatant, measured with the DCIP and the cyt *c* assay reached a maximum value of 376 and 320 U L^{-1}, respectively. The concentration of soluble protein in the culture supernatant increased to 633 mg L^{-1}. After 111 h the

cultivation was stopped since the specific activity in the culture supernatant started to decline. The recombinant enzyme was purified to homogeneity using a two-step purification protocol (Supporting information, Table S1). Strict pooling of the purest fractions resulted in a moderately high yield of 71% and 295 mg of a homogenous enzyme preparation. The absorbance ratio A_{420}/A_{280} was 0.65. The homogeneous *rCt*CDH preparation had a specific activity of 2.84 U mg^{-1} (measured with the cyt *c* assay) and 4.1 U mg^{-1} (measured with the DCIP assay).

3.2 Molecular properties

The *rCt*CDH and *Ct*CDH were comparatively characterized. Molecular masses were determined by SDS-PAGE (Supporting information, Fig. S1). The *rCt*CDH showed a broad and diffuse band between 89 and 96 kDa whereas *Ct*CDH was smaller and less diffuse (83 ± 3 kDa). After deglycosylation under denaturing conditions with PNG-ase F, single, sharp bands with an identical molecular mass of 78 kDa were found for both enzymes. The UV/Vis spectra of the purified enzymes (Figs. 2A and B) are characteristic for CDHs. Based on the two obtained spectra the amount of the bound FAD cofactor was estimated. The calculated molar absorption coefficient of *Ct*CDH at 280 nm (ε_{280} = 149.03 mM^{-1} cm^{-1}) was used for the determination of the protein concentration. After protein precipitation with trichloroacetic acid [17] the amount of the released FAD was calculated using the molar absorption coefficient for free FAD (ε_{450} = 11.3 mM^{-1} cm^{-1}). From the obtained values, it was calculated that 92% of the active sites in *Ct*CDH contain FAD, but only 30% in *rCt*CDH.

3.3 Kinetic properties

Catalytic constants of *rCt*CDH and *Ct*CDH were measured with cyt *c* as electron acceptor at pH 7.4 for cellobiose and glucose (Table 1). The K_M values of the tested electron donors were similar for both CDHs. Both CDHs exhibited very low K_M values for cellobiose (~8 µM). The K_M values for glucose are about 11 500-fold higher (~90 mM). In contrast to the almost identical K_M values, a systematic difference between the two CDH preparations was observed for the k_{cat} values, which were ~5 times lower for *rCt*CDH. The pH-dependent activities with DCIP and cyt *c* as electron acceptors were measured (Figs. 3A and B). The relative pH profiles of *Ct*CDH and *rCt*CDH match perfectly. However, the specific activities for cyt *c* at the pH optimum (7.5) of the two preparations were 3.2 and 14.7 U mg^{-1}, respectively. The same trend can be seen for DCIP at the pH optimum (5.0) with 10.5 and 39 U mg^{-1}.

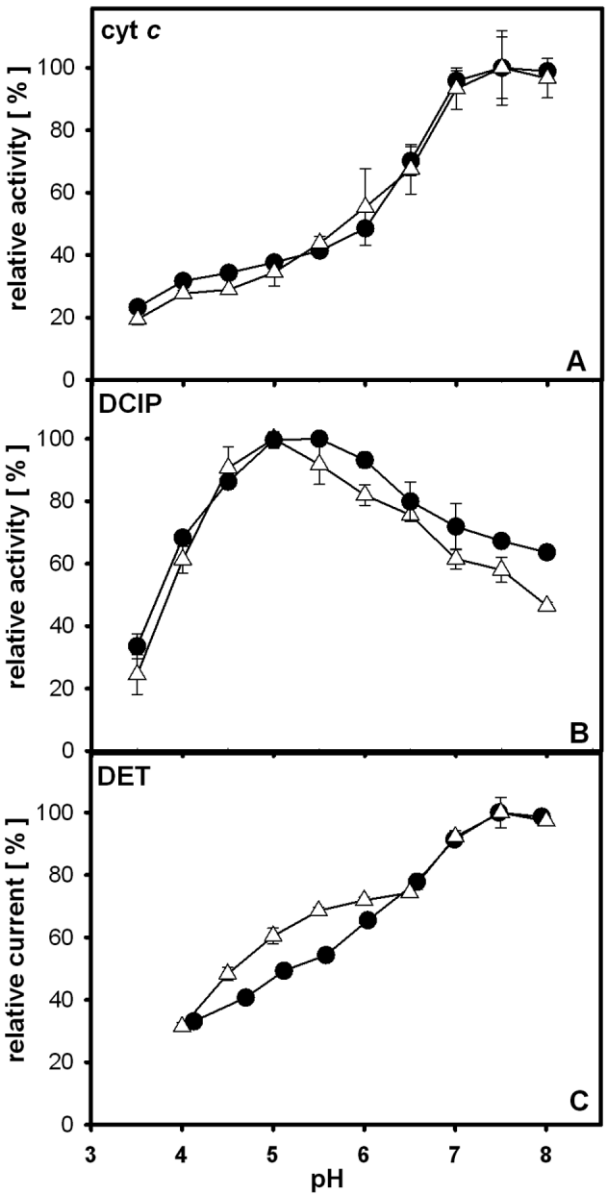

Figure 3. Comparison of the pH-dependent reduction of DCIP (**A**), cyt *c* (**B**) and direct electron transfer (DET) to a graphite electrode (**C**) of *Ct*CDH (circles) and *rCt*CDH (triangles).

3.4 Electrochemical characterization

The interaction of *rCt*CDH with self-assembled monolayers (SAM)-modified gold electrodes was investigated by cyclic voltammetry. Direct communication of CYT_{rCtCDH} with the electrode was found and characterized. CYT_{rCtCDH} exhibited a midpoint potential ($E_{1/2}$) of 100 mV vs. normal hydrogen electrode (NHE), determined by cyclic voltammetry (Fig. 4A) and square wave voltammetry (Fig. 4C). The peak separation between the anodic and cathodic peak potentials of the cyclic voltammogram was 60 mV. The presence of the DH_{CDH} domain was proven by

Table 1. Comparison of steady-state kinetic constants of *Ct*CDH and *rCt*CDH using cyt *c* as electron acceptor in 100 mM Mc Ilvaine buffer, pH 7.5

Carbohydrate	Enzyme	K_M [μM]	k_{cat} [s^{-1}]	k_{cat}/K_M [M^{-1} s^{-1}]
Cellobiose	*Ct*CDH	7.8 ± 0.6	13.0 ± 0.7	1.7×10^6
	*rCt*CDH	7.9 ± 0.5	2.0 ± 0.1	2.5×10^5
Glucose	*Ct*CDH	93000 ± 6500	12.8 ± 1.4	1.40×10^2
	*rCt*CDH	88000 ± 4000	2.2 ± 0.2	2.50×10^1

measuring the catalytic current in the presence of 50 mM glucose (Fig. 4B), which resulted in a maximal catalytic current of 30.3 μA cm^{-2} at 300 mV vs. NHE. The pH-dependent DET current of *rCt*CDH-modified spectrographic graphite electrode was measured in the presence of 50 mM glucose in a flow cell (Fig. 3C). The relative pH profiles of *rCt*CDH and *Ct*CDH resemble each other well, but *rCt*CDH has a higher relative catalytic current at low pH. The specific catalytic current in the presence of 50 mM glucose was 4.39 μA cm^{-2} for *Ct*CDH and 1.47 μA cm^{-2} for *rCt*CDH.

4 Discussion

The expression cassette with the native signal sequence controlled by the AOX elements led to the successful expression of *Ct*CDH in *P. pastoris*. The protein concentration of *rCt*CDH reached by fermentation was 132 mg L^{-1}. This is, when compared to the other heterologously expressed CDHs not extraordinary high, but satisfactory. The applied purification procedure resulted in a homogeneous *rCt*CDH preparation. The homogeneity of the protein is, however, compromised by the sub-stoichiometric occupation of the cofactor-binding site by FAD. Since the activity of *rCt*CDH during purification and in homogeneous state is stable over a wide pH range, it does not seem that FAD is lost during the cultivation, purification or storage. It is probable that the FAD is not incorporated into the enzyme during protein synthesis. The generally lower specific activities of recombinantly produced CDHs in *P. pastoris* point in this direction [18–22]. Only 30% of the *rCt*CDH molecules have FAD incorporated, which is three-fold lower than that found for *Ct*CDH (92%). A probable reason for this low FAD occupation might be the high expression rates of other FAD-dependent enzymes (e.g. alcohol oxidase) in *P. pastoris* during induction with methanol. However, supplementation of the fermentation media with riboflavin did not increase the FAD content in *rCt*CDH (data not shown).

The simultaneous characterization of *rCt*CDH and *Ct*CDH shows besides a number of similarities also differences between the two enzymes. The SDS-PAGE shows a higher amount of glycosylation for *rCt*CDH while the

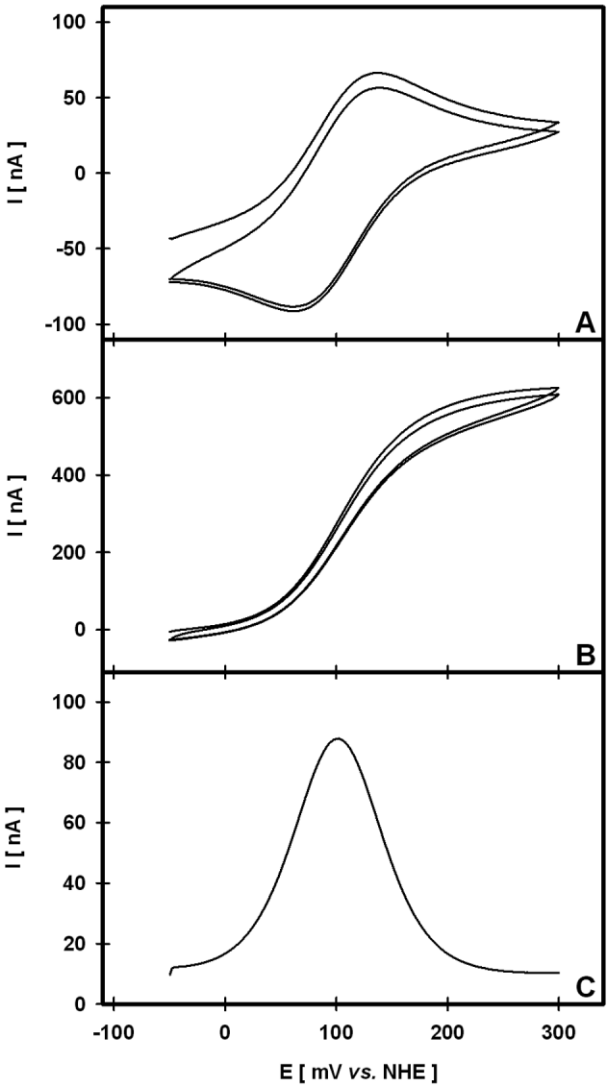

Figure 4. Electrochemical characterization of recombinant *rCt*CDH. Non catalytic (**A**), catalytic (50 mM glucose, **B**) and square wave (**C**) voltammograms of CDH (5 mg mL^{-1} final concentration) on thioglycerol-modified gold electrodes entrapped under a permselective membrane.

deglycosylated enzymes have the same molecular mass. In this case, *P. pastoris* seems to hyperglycosylate the six potential N-glycosylation sites in *rCt*CDH. The spectral properties are similar with regard to the absorption maxima, but differ in the relative ratios of the absorbance. It is obvious from Fig. 3 when looking at the shoulder between 450 to 500 nm, that the concentration of FAD in *rCt*CDH is lower than that in *Ct*CDH.

The pH profiles of *rCt*CDH and *Ct*CDH are remarkably similar regarding their relative activities (activities normalized by the highest activity at the pH optimum). Both have their pH optimum with cyt *c* at 7.5 and with DCIP at 5.0. However, there is a difference in the actual turnover numbers. For cyt *c* *rCt*CDH has a k_{cat} of 3.22 s^{-1} while

*Ct*CDH has a k_{cat} of 14.75 s^{-1}. In case of DCIP k_{cat} is 10.5 s^{-1} and 39 s^{-1}, respectively. In both cases the ratio of *rCt*CDH to *Ct*CDH turnover numbers (0.22 and 0.27) resembles the ratio of FAD carrying CDH molecules (0.30). The trend was also observed in steady-state kinetic measurements of *rCt*CDH and *Ct*CDH using different carbohydrates and electron acceptors. The k_{cat} values were ~5 times lower for *rCt*CDH. It is generally found that recombinantly produced CDHs have a lower specific activity, but it is not investigated if the reason comes only from FAD depletion or also from other factors, e.g. different glycosylation patterns or changes in the protein conformation. Although rate constants differ, the obtained K_M values are very similar, indicating that the active fraction of *rCt*CDH has the same catalytic properties as *Ct*CDH. This FAD depletion seems to be the main reason for the reduced catalytic activity of *rCt*CDH. Additional proof was obtained from reconstitution experiments with FAD where the turnover number of *rCt*CDH was increased 1.5 times. The reconstitution was, however, incomplete.

The midpoint potential of the heme *b* in CYT_{rCtCDH} (100 mV vs. NHE) is in good agreement with the previously estimated value for *Ct*CDH (90 mV vs. NHE, [10]) and suggests that the heme *b* is incorporated in the same way as in *Ct*CDH. The difference in the oxidation and reduction peak in the cyclic voltammogram is very close to the theoretical value of 59 mV, indicating a reversible electron transfer process between the CYT_{CDH} and the gold electrode. The catalytic functionality of the *rCt*CDH on the thiol- modified gold electrode was proven by measuring catalytic current in the presence of lactose. A thiol glycerol-modified gold surface was found to be an efficient support for contacting the *rCt*CDH and resulted in high catalytic currents (30.3 µA cm^{-2} at 300 mV vs. NHE) under turnover conditions in the presence of 50 mM glucose. The low midpoint potential of *Ct*CDH makes it possible to operate the biosensor at 200 mV vs. NHE, where the maximum current is already reached. A low operational potential minimizes the unspecific oxidation of interfering substances. The specific catalytic current found for spectrographic graphite electrodes at a 50 mM glucose concentration was much lower (4.39 µA cm^{-2} at 405 mV vs. NHE) despite the higher applied potential. This is either a result from a lower surface coverage of CDH on the graphite electrode or a less efficient DET. The influence of the pH on the catalytic current was similar for both the *Ct*CDH and the *rCt*CDH. The maximum specific catalytic current for *rCt*CDH and for *Ct*CDH was obtained for both at pH 7.5.

The *rCt*CDH shows in large part the same properties as *Ct*CDH, which was successfully used as biocatalyst for glucose biosensors and glucose fueled biofuel cell anodes [23–25], and can efficiently communicate via DET with either thiol-modified gold electrodes or spectrographic graphite electrodes at physiological pH. The Michaelis-Menten constants for carbohydrates and electron acceptors are not changed and the redox potential is the same for *rCt*CDH and *Ct*CDH. The low FAD occupation in the active site of *rCt*CDH, which reduces the specific activity of the homogeneous enzyme and the obtained current density on electrodes is, however, a major point of further improvement.

It can be concluded that the successful recombinant expression of *Ct*CDH and its proven applicability are important steps towards its use in bioelectronics, but there is need for further optimization. Further research will focus on the optimization of the cofactor loading as well as genetic engineering of the substrate specificity of *rCt*CDH.

The authors thank the European Commission (project "3D-Nanobiodevice" NMP4-SL-2009-229255) for financial support.

The authors declare no conflict of interest.

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