Direct Electron TransferA Favorite Electron Route for Cellobiose Dehydrogenase (CDH) from Trametes v illosa . Comparison with CDH from Phanerochaete c hrysosporium †

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Direct Electron Transfer—A Favorite Electron Route for Cellobiose Dehydrogenase (CDH) from *Trametes villosa*. Comparison with CDH from *Phanerochaete chrysosporium*†

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This paper presents some functional differences as well as similarities observed when comparing the newly discovered cellobiose dehydrogenase (CDH) from *Trametes villosa* (*T.v.*) with the well-characterized one from *Phanerochaete chrysosporium* (*P.c.*). The enzymes were physically adsorbed on spectrographic graphite electrodes placed in an amperometric flow through cell connected to a flow system. In the case of *T.v.*-CDH-modified graphite electrodes, a high direct electron transfer (DET) current was registered at the polarized electrode in the presence of the enzyme substrate reflecting a very efficient internal electron transfer (IET) process between the reduced FAD-cofactor and the oxidized heme-cofactor. In the case of *P.c.*-CDH-modified graphite electrodes, the DET process is not as efficient, and the current will greatly increase in the presence of a mediator (mediated electron transfer, MET). As a consequence, when comparing the two types of enzyme-modified electrodes an inverted DET/MET ratio for *T.v.*-CDH is shown, in comparison with *P.c.*-CDH. The rates of the catalytic reaction were estimated to be comparable for both enzymes, by measuring the combined DET + MET currents. The inverted DET/MET ratio for *T.v.*-CDH-modified electrodes might suggest that probably there is a better docking between the two domains of this enzyme and that the linker region of *P.c.*-CDH might have an active role in modulating the rate of the IET (by changing the interdomain distance), with respect to pH. Based on the new properties of *T.v.*-CDH emphasized in the present study, an analytical application of a third-generation biosensor for lactose was recently published.

Introduction

To understand biological electron transfer (ET) pathways, which may possibly serve as models for the design of ET pathways in biosensor applications, the specific features of the ET pathways have to be elucidated. Biosensors based on direct electron transfer (DET) are of particular importance because of the absence of any mediator, supposedly providing them with superior selectivity due to prevention of interfering reactions and the lack of yet another reagent in the reaction sequence. However, DET has been proven only for a restricted number of redox enzymes, and the majority of such redox enzymes were shown to contain metallocenters, with heme in most cases.[1−7] One of these multiple-domain enzymes containing one catalytic domain and additionally a heme domain as a "built in electron-transfer mediator" is cellobiose dehydrogenase.

Cellobiose dehydrogenase (EC 1.1.99.18; CDH) is an extracellular enzyme produced by a variety of fungi, such as white rot, brown rot, and plant pathogen fungi.[8,9] CDH from white rot has a proposed role in the early events of lignocellulose degradation and wood colonization. In the catalytic reaction supported by white rot CDH, the C1 carbon of cellobiose and higher cellooligosaccharides (supposed to be the natural substrates) is oxidized with the formation of the corresponding lactones. Additionally, some other disaccharides that have a β-1-4-linkage with a β-glucose at the reducing end (e.g., lactose) can be oxidized by CDH.[10]

The enzyme is a monomer, consisting of two separate domains, one domain containing FAD as cofactor and the other domain harboring a cytochrome *b*-type heme. The two domains are kept together by a peptide linker region, which in the case of *Phanerochaete chrysosporium* CDH (*P.c.*-CDH), is characterized by a high content of hydrophilic amino acids (i.e., threonine and serine).[8] The linker region is the site for a proteolytic cleavage with papain but also indigenous proteases that results in the separation of the two domains.[11] Each domain presents a different isoelectric point, viz. 3.42 for the heme domain and 5.45 for the FAD domain of *P.c.*-CDH.

The crystal structures of both individual domains for *P.c.*-CDH have been separately elucidated.[12,13] The X-ray structure

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of the heme domain shows that the heme is ligated by a His/Met couple, which is unique among the cytochrome-containing enzymes, and that the heme is docked at the exterior of the enzyme, having propionyl groups oriented toward the surrounding solvent.[13]

In the oxidation reaction of cellobiose at the FAD domain, two electrons and two protons are transferred to the FAD-cofactor. These electrons are then further delivered either to a two-electron acceptor that reacts directly at the FAD domain (e.g., quinones) or to a one-electron acceptor (e.g., ferricenium ion, cytochrome *c*) via the heme domain. To involve the heme cofactor in the reaction with a one-electron acceptor, the electrons must be sequentially transferred one by one from the FAD domain to the heme domain, via an internal electron transfer (IET) process.

In the past few years, *P.c.*-CDH was extensively characterized using electrochemical investigations. By the entrapment of *P.c.*-CDH under a dialysis membrane, the enzyme was maintained in the proximity of thiol-based self-assembled monolayer (SAM) modified Au electrodes, and it showed a facile electron-transfer reflecting the electrochemistry of the $Fe^{2+/3+}$-redox couple of the heme domain.[14] In the presence of cellobiose, a bioelectrocatalytic current (CAT) was exhibited. Additionally, it was also proven that the heme domain is responsible for the direct electronic communication (DET) of the enzyme with the SAM-Au electrode,[15] whereas the FAD domain was never shown to be able to directly exchange electrons with the electrode. However, through the recycling of a two-electron acceptor (e.g., a quinone) used as a mediator between the reduced FAD domain (site for mediator reduction) and the electrode (site for mediator oxidation) an efficient electron transfer is achieved, known as mediated electron transfer (MET).[16]

When CDH was adsorbed on a graphite electrode and used as a detector in a flow injection system, different response peaks for DET (that includes IET and CAT, too) and MET were experimentally obtained and are exemplified in Figure 1 and described in detail elsewhere.[16,17] Briefly, the injection of a sugar substrate into the carrier buffer results in the appearance of DET peaks due to the flow of electrons through the DET route, as schematically represented in the insert of Figure 1. A continuous addition of a certain concentration of sugar to the carrier buffer will lead to a steady-state DET current. If now instead samples containing 1 μM hydroquinone will be injected into the carrier containing both sugar and buffer, on top of the steady-state DET current response peaks to hydroquinone will appear as a result of that the same hydroquinone molecule will be oxidized multiple times at the electrode due to its continuous regeneration into its reduced state by the reduced FAD cofactor of CDH, leading to MET currents, as illustrated in Figure 1.

Recently, among a series of fungi that were identified to produce CDH, the white-rot fungus *Trametes villosa* was seen as an excellent candidate for CDH production yielding high activity. *T.v.*-CDH has a molecular mass of 90 kDa and an isoelectric point of 4.2−4.4. From biochemical characterization of *T.v.*-CDH, cellobiose shows a K_m of 210 μM and a k_{cat} of 24 s^{-1}. A very high activity with one-electron acceptors, such as ferricenium and ferricyanide, was also reported.[18] Neither the amino acid sequence nor the crystal structure of *T.v.*-CDH is known. Cyclic voltammetry was used to investigate the interaction of *T.v.*-CDH with Au electrodes modified with SAMs using different alkenothiols.[19] The enzyme showed a very good DET reaction with the SAM-Au electrodes, and the effect of pH and the type of the functional headgroup of the alkenothiols were seen to play an important role for the orientation of the enzyme on the electrode, with considerable implication for both the efficiency of the DET and the bioelectrocatalytic current.

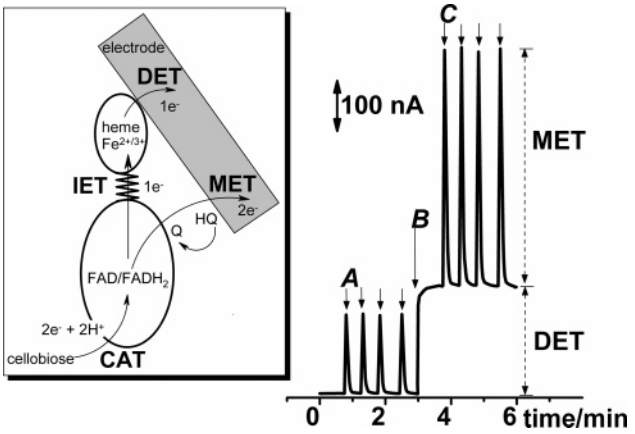

Figure 1. Example of experimental peaks that illustrate how DET and MET currents were measured: (A) injection of 200 μM sugar into the carrier buffer; (B) continuous supply of 200 μM sugar in the carrier buffer that leads to a steady-state DET current; (C) injection of 1 μM hydroquinone into the (200 μM sugar + buffer) carrier that results in MET current peaks on top of steady-state DET current. Insert: electron-transfer pathways for CDH responsible for electronic communication between CDH and the electrode. Abbreviations: CAT, catalytic reaction; IET, interdomain electron transfer; MET, mediated electron transfer; DET, direct electron transfer.

Potential applications of CDH were exploited in bioremediation for the degradation of recalcitrant pollutants,[20] in biotechnology for the preparation of organic acids[21] and for the production of lactose-free galacto-oligosaccharide mixtures,[22] and in biosensor development. Two types of biosensors, using *P.c.*-CDH, were previously developed based on two different principles: (i) MET (second generation biosensors) for detection of catecholamines[16] and quinones;[17] and (ii) also MET via the heme cofactor using $Os^{2+/3+}$ complex containing polymers for detection of disaccharides, such as cellobiose and lactose.[23−25] Recently, our group presented the analytical application of newly discovered sources of white rot CDH (*Trametes villosa* and *Phanerochaete sordida*) for detection of lactose, and the resulting biosensors showed a linear response range in the low micromolar concentration range (1−100 μM) that is far below the detection limits of all previously published biosensors for lactose.[26]

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This work comes to emphasize the major differences of the newly discovered *Trametes villosa*-CDH (*T.v.*-CDH), in comparison with the well studied *Phanerochaete chrysosporium*-CDH (*P.c.*-CDH) and to suggest an explanation for the high efficiency of DET for the first one. In this respect, we examined the relation between the different electron-transfer pathways available in *T.v.*-CDH using a graphite electrode as an artificial electron acceptor for the heme domain and also as a regenerator of an added two-electron acceptor that is reduced at the FAD domain, as illustrated in insert of Figure 1. In comparison to the well-studied *P.c.*-CDH, *T.v.*-CDH proves to have the DET route as the most favorable ET pathway to the electrode. The observed differences in the efficiencies of each ET pathway for each enzyme might be attributed to possible discrepancies in the protein sequence that connects the FAD and heme domains (linker region) with each other. The importance of such a linker region between the domains of a protein for the interdomain electron-transfer processes was demonstrated by White et al. in 1993, using site-directed mutation of flavocytochrome b_2.[27]

Materials and Methods

Reagents. The following chemicals were of analytical grade (> 97% purity) and used as received: hydroquinone (HQ/Q) from Acros (Geel, Belgium) and β-D-(+)-cellobiose (S) and β-D-(+)-lactose from Sigma (St. Louis, MO). All aqueous solutions were prepared using water (18 mΩ conductance) purified with a Milli-Q system (Millipore, Bedford, MA). A stock solution of hydroquinone (10 mM) was prepared in water right before use and further diluted in the carrier solution to the necessary concentrations. Two types of buffers were used: type A, 20 mM sodium-acetate buffer at pH 4.5; and type B, 20 mM phosphate-citrate buffer at pH 6. Buffers were filtered through a 0.22 μm porous membrane (Millipore) followed by vacuum degassing before use.

Two different cellobiose dehydrogenases (CDH) were used, viz. from *Phanerochaete chrysosporium* (provided by Dr. Gunnar Henriksson, The Royal Institute of Technology, Stockholm, Sweden) as a solution of 0.5 g·L^{-1} in sodium-acetate buffer, pH 5, purity 95% and from *Trametes villosa* CBS 334.49 (purified at University of Natural Resources and Applied Life Sciences Vienna, Austria, as a solution of 2.3 g·L^{-1} and of similar purity).

Enzyme-Modified Electrode. CDH was immobilized by simple chemo-physical adsorption in a random orientation onto the surface of a solid spectroscopic graphite rod (Ringsdorff Werke GmbH, Bonn, Germany, Type RW001, 3.05 mm Ø). The roughness of the graphite electrode is considered to be 5. The electrode was previously polished on wet fine emery paper (Tufbak Durite, P1200), followed by careful rinsing with Milli-Q water. A volume of 5 μL of CDH solution was spread onto the entire active surface of the electrode. The electrode was allowed to dry at room temperature, and then it was stored overnight in a refrigerator (4 °C). The electrode is stored overnight (or for more than 8 h) in the refrigerator, to facilitate a stronger adsorption/interaction of the enzyme molecule with the electrode surface and consequently to obtain a better uniform adsorption of enzyme molecules on the electrode. Previous experiments have shown that this resting time improves the performances of the electrode. Before use, any weakly adsorbed enzyme on the electrode was removed by careful rinsing with Milli-Q water.

Flow Injection Setup. An amperometric flow through cell of the wall jet type was used containing three electrodes: the working electrode (the CDH-modified graphite electrode), a reference electrode (Ag|AgCl in 0.1 M KCl), and a platinum wire as the auxiliary electrode. The potential of the working electrode was maintained constant at +300 mV versus the reference electrode using a potentiostat (Zäta Elektronik, Lund, Sweden). The resulting current was recorded on a strip chart recorder (Kipp and Zonen, Delft, The Netherlands). The electrochemical cell was connected on-line to a single line flow injection (FI) system, in which the carrier flow was maintained at a constant flow rate of 0.5 mL·min^{-1} by a peristaltic pump (ALITEA, Stockholm, Sweden). An electrically controlled six-port valve (LabPRO, Rheodyne, Cotati, CA) with a 50 μL injection loop was used as injector. Each measurement is the average of four different injections and the standard deviation was 0.2%.

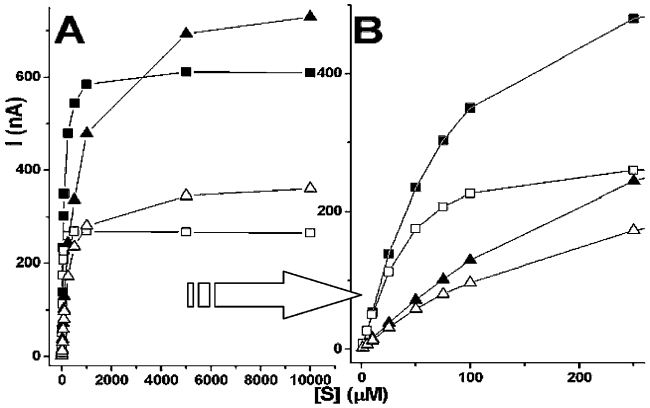

Figure 2. (A) Current versus substrate concentration (Michaelis−Menten) plots for a *T.v.*-CDH-modified graphite electrode obtained by measuring the resulting DET using different substrates, lactose (triangles) and cellobiose (squares), at pH 4.5 (filled symbols) and at pH 6.0 (open symbols). (B) The linear domains in the low micromolar range for cellobiose (squares) and lactose (triangles) at pH 4.5 (filled symbols) and pH 6.0 (open symbols).

Results and Discussions

The kinetic parameters of adsorbed *T.v.*-CDH were determined through injection of either of the main substrates, cellobiose or lactose, into the carrier buffer. In this mode, the resulting signals for the substrate correspond to the direct electron transfer (DET) process that implicitly includes also the catalytic reaction (CAT) and the internal electron transfer (IET) from FAD_{red} to $heme_{ox}$, as depicted in Figure 1. The resulting dependence of the DET currents on the concentration of substrate is shown in Figure 2A. It can be observed that both substrates obey an apparent Michaelis−Menten dependence for the DET currents. However, a closer inspection of the plots (see Figure 2B) reveals an intriguing kinetic behavior for cellobiose but not for lactose that was also previously observed for *P.c.*-CDH[10,28] (i.e., substrate inhibition). This means that, even though cellobiose presents a higher initial catalytic rate than lactose, the maximum rates of the catalytic reaction are obtained for lactose. In other words, even though cellobiose has a smaller K_m than lactose, equivalent to a higher affinity of the enzyme for cellobiose, however, a higher k_{cat} value is registered in the case of lactose. The observed effect is proven for *P.c.*-CDH to appear due to substrate inhibition caused only by cellobiose.[28]

To demonstrate that also *T.v.*-CDH presents the inhibition effect only for cellobiose, but not for lactose, the plots in Figure 2B were re-plotted to their corresponding Lineweaver−Burk (LB) representations, as presented in Figure 3. The resulting LB plots were linearly fitted twice, once for the lowest concentration range and second for the range of the highest concentrations of substrate. In the case of lactose (Figure 3A), the resulting fitting lines obtained at constant pH were overlapping, which means that the enzyme obeys the same kinetic equation with the same parameters, for both low and high lactose concentrations. In

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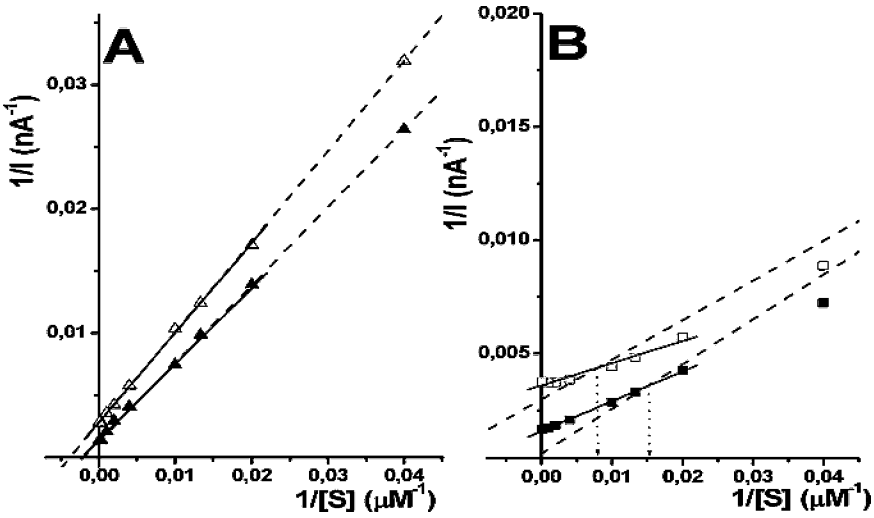

Figure 3. Lineweaver−Burk plots corresponding to Figure 2A (zoomed on the range of high concentrations of substrates) show a partial inhibition caused by cellobiose (Figure 3B) and no inhibition effect by lactose (Figure 3A). Dashed fitting lines correspond to low concentrations of substrate and solid fitting lines correspond to high concentrations of substrate. Experimental conditions: filled marks (pH 4.5) and open symbols (pH 6.0).

addition, this suggests that no inhibition effect is seen for lactose, irrespective of the experimental pH.

However, the resulting two fitting lines for cellobiose show two different domains of linearity, which means that the rate of the catalytic reaction is decreased for a higher concentration of cellobiose, which is equivalent to a *partial inhibition* effect of cellobiose. From the intersection of these two linear domains, it was calculated that, at pH 4.5, *T.v.*-CDH is inhibited at concentrations of cellobiose higher than 65 μM, whereas at pH 6.0, the enzyme switches to a lower catalytic rate for concentrations of cellobiose higher than 125 μM. This is in accordance with a previous report on *T.v.*-CDH, where the inhibiting effect of cellobiose was observed at concentrations higher than 70 μM at pH 4.5 using ABTS radical as a 1 e^- acceptor.[18]

However, it should be emphasized that the registered DET current (or activity using a 1 e^- acceptor) reflects the involvement of three steps in the electron-transfer pathway, viz., CAT, IET, and DET, see Figure 1. In our previous investigation on the inhibition effect of cellobiose seen for *P.c.*-CDH,[28] it was proven that in fact cellobiose does not affect the catalytic activity, but the decrease in the rate of IET is due to the formation of a complex [$FADH_2$/H$^\bullet$-S] between one of the reduced forms of the FAD (viz., $FADH_2$ or $FADH^\bullet$ that is formed after the transfer of the first electron from $FADH_2$ to the oxidized heme) and excess of cellobiose (S). It was demonstrated that this inhibition effect could be overcome by a high concentration of quinones, meaning that cellobiose is seen as a *competitive inhibitor for quinone*, as could be evaluated by measuring the MET current. On the other hand, the heme domain is not involved in any competition with cellobiose for the entrance to the active site of the enzyme, as was previously suggested by Hallberg et al.,[12] who proposed that the propionyl groups surrounding the heme protrude into the active site of CDH to pick-up the electrons from the reduced FAD domain. Otherwise the formation of a [$FADH_2$/H$^\bullet$-S] complex, which was proven by Cameron and Aust using ESR spectroscopy,[29] must be seen as the formation of a dead-end complex that must drastically affect the IET. Here by measuring DET currents, the inhibition effect of cellobiose appears only as a *partial inhibition* of the IET due to probably some thermodynamic limitations (changing the reorganization energy for the IET process).

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Table 1. Apparent Kinetic Parameters for *T.v.*-CDH-Modified Electrodes Calculated from Figure 2A (Confidence Interval of 95%)

	pH = 4.5		pH = 6.0	
substrate	lactose	cellobiose	lactose	cellobiose
V_{max} (nA)	764.29 ± 30.17	626.02 ± 9.97	365.87 ± 3.17	279.70 ± 11.60
K_m (μM)	553.25 ± 75.78	81.41 ± 5.42	277.69 ± 10.65	31.80 ± 6.54
V_{max}/K_m	1.38	7.69	1.32	8.79

As can be understood from the results shown in Figures 2 and 3, not only does an increase in the concentration of cellobiose produce a limited rise of the DET currents but an increase in pH has an even more pronounced effect; it will cause a decrease in the efficiency of DET. Irrespective of the type of substrate, an increase in pH produces a parallel upshift (same slopes, but higher intercepts) of the LB plots (see Figure 3) along the *Y* axis that corresponds to lower K_m values, lower V_{max} values, and almost identical slopes of the Lineweaver−Burk plots. The parameters K_m and apparent V_{max} (measured as maximum DET current) were calculated from the plots in Figure 2A, and their values are presented in Table 1. This parallel upshift is valid for the entire range of the plot, even for the inhibition regions in the case of cellobiose. Such a shift effect is equivalent to an *uncompetitive* inhibition of the DET currents caused by an increase in pH. Once more, it should be pointed out that the catalytic activity is not affected by an increase in pH from 4.5 to 6.0, as was also shown in another report,[16] where it was observed that the highest biocatalytic current was recorded at pH 6.0 by measuring the MET current at a *P.c.*-CDH-modified graphite electrode.

As a suggestion from the influence of pH, it could be introduced the hypothesis that the variation of the DET currents with pH observed at various CDH-modified electrodes could in fact be the result of an adjustment of the distance between the two domains of the enzyme due to electrostatic forces and in which the linker region plays the role of a "rope". The electrostatic potentials at the surfaces of each domain of *P.c.*-CDH are illustrated in Figure 4, and they were calculated using SwissPdbViewer (http://www.expasy.org/spdbv/) and taking the partial atomic charge into consideration. It can be seen that both domains have dominant negative electrostatic potentials, confirmed also by the isoelectric points of each domain. However, the FAD domain presents a close accumulation of a positive

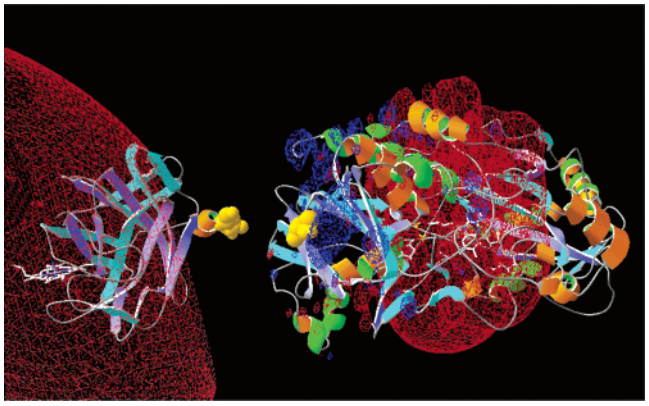

Figure 4. Calculation of electrostatic potentials at the surfaces of the heme domain (PDB-file 1D7D) (left side of the picture) and the FAD domain (PDB-file 1NAA) (right side of the picture) of *P.c.*-CDH, using SwissPdbViewer, illustrating a "sandwich-like conformation" of electrostatic forces between the two domains. Electrostatic maps correspond to atomic partial charges: the red-map corresponds to negative electrostatic potentials and the blue-map corresponds to positive electrostatic potentials. The amino acids drawn in balls-mode represent the connections of each domain to the linker region: for the FAD domain it is THR215 and for the heme domain it is ASN183.

electrostatic potential around the region of connection to the linker region. On the other hand, the entire heme domain produces a strong negative electrostatic potential. From these considerations, it is expected that the two domains are docked to each other in a "sandwich-like conformation". An interesting observation is also the fact that the histidine residue that coordinates the iron of the heme-group is just the tenth amino acid from the linker region, being separated from the linker region by only a beta strand. Is the short connection of the histidine to the linker region offering to the linker region a modality to adjust the formal potential of the heme cofactor and the position in which the heme domain interacts with the FAD domain? This is an important question that is still waiting to be proven by experiments.

As a conclusion, the electrostatic forces between the domains are responsible for the adjustment of the interdomain distance (rope-folding/unfolding) that leads to a modulation of the rate of the IET from the FAD domain to the heme domain. According to this, the interdomain distance should become minimum at pH 4.5, at which the optimum DET is observed, and maximum at pH 6.0, at which the enzyme preserves in principle the MET function.

Up to here, the similarities in response between the *T.v.*-CDH- and *P.c.*-CDH-modified electrodes have been shown, regarding the inhibition effect by cellobiose (not by lactose) and the influences of pH on DET/IET. However, the most interesting aspect of the newly discovered *T.v.*-CDH is the difference to *P.c.*-CDH, namely that the two enzymes have an inverted ratio of the DET:MET relationship. To prove this statement, DET and MET were recorded for both lactose and cellobiose as substrate at a low (4.5) and at a high (6.0) pH. The values of the MET and the DET currents are presented in Figure 5, for each of the above cases, with the specification that the MET currents were measured by injection of 1 μM hydroquinone into the carrier also containing 200 μM sugar substrate and registering the response on top of steady-state DET current. In the case of *P.c.*-CDH, in accordance with previous reports,[16,28] the DET current takes its maximum value at pH 4.5 and it decreases by 50% at pH 6.0, whereas the maximum MET current is observed at pH 6.0 at which a nine times higher value is observed compared with the one at pH 4.5. At pH 6.0, the highest catalytic rate for

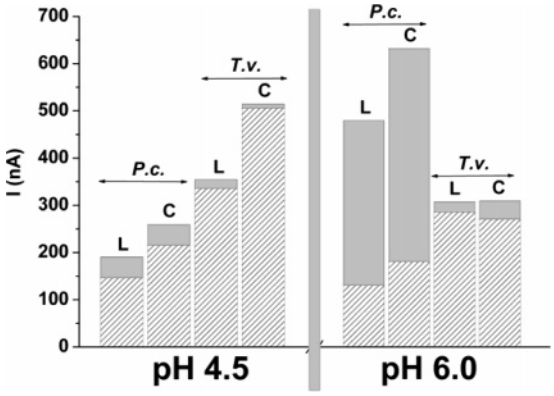

Figure 5. Comparison of the steady-state DET (lined columns) currents for 200 μM sugar and the MET (grey columns) currents for injection of 1 μM hydroquinone obtained at *P.c.*-CDH- and *T.v.*-CDH-modified electrodes, with respect to cellobiose (C) and lactose (L), and to pH (4.5 or 6.0). The MET values represent the average of four consecutive injections with a standard deviation of below 0.2%.

P.c.-CDH is measured as the sum of both the DET and the MET currents. These observations are valid for both the two substrates, cellobiose (inhibiting substrate) and lactose (noninhibiting substrate), of the enzyme.

Surprisingly, however, in the case of *T.v.*-CDH, the efficiency of IET, by measuring DET, is so high that the 2 e^- acceptor is virtually eliminated from reacting at the FAD domain. This means that the IET process is a strong competitive route to the MET process. Thus, DET currents recorded at pH 4.5 are 60% higher at the *T.v.*-CDH-modified electrode compared with those at the *P.c.*-CDH-modified electrode, whereas the MET currents are almost nonexistent for the *T.v.*-CDH-modified electrode. Even when the pH is increased to 6.0, the MET current does not reach more than 10% in relation to the MET obtained in the case of *P.c.*-CDH. The DET currents, recorded with the *T.v.*-CDH-modified electrode at pH 6.0, decrease by 50% for cellobiose and by only 20% for lactose from the values obtained at pH 4.5. This preservation of the enzymatic activity over an extended pH range was also previously observed in the case of *T.v.*-CDH at SAM-modified Au electrodes.[19] Another observation for the *T.v.*-CDH-modified electrodes is that at pH 6.0 the DET current for lactose turns to be higher than the one registered for cellobiose, in contrast to the situation at pH 4.5, which suggests that an increase in pH contributes much more to the importance of the inhibition effect of cellobiose (also shown previously by Igarashi et al.[30]) than to the limitation of the rate of the catalytic reaction at the FAD domain.

It is also observed that the DET currents obtained for the *T.v.*-CDH-modified electrode at pH 6.0 are even higher than the DET currents obtained for *P.c.*-CDH-modified electrodes at pH 4.5. So, by taking into consideration that the catalytic rate for *P.c.*-CDH is higher than the catalytic rate for *T.v.*-CDH (by comparing the sum of DET+MET currents), it can be concluded that *T.v.*-CDH has a much higher rate of IET at pH 6.0 compared with the IET rate obtained for *P.c.*-CDH at pH 4.5 (optimum pH value for a 1 e^- acceptor process).

The conclusion from these observations is that the sequential electron-transfer processes of CAT-IET-DET constitutes the favorite route of the electrons from the substrate to the electrode in the case of *T.v.*-CDH, due to a much more favorable IET process. In contrast, the main route of the electrons in the case of *P.c.*-CDH involves only the CAT and MET steps.

(30) Igarashi, K.; Momohara, I.; Nishino, T.; Samejima, M. *Biochem. J.* **2002**, *365*, 521−526.

A possible explanation for the difference in the favorite ET routes between *T.v.*-CDH and *P.c.*-CDH might be that in the case of *T.v.*-CDH the two domains are better docked to each other due to a shorter linker region or because the region of the positive electrostatic potential is more widely spread on the surface of the FAD domain that would make the IET process more favorable due to less specific localization of attraction between the domains. This might imply that the FAD domain of *T.v.*-CDH has to have a higher isoelectric point in comparison to the one from *P.c.*-CDH.

This major difference between the enzymes confers new opportunities for analytical application for *T.v.*-CDH, due to the excellent DET properties and its use for the development of third-generation biosensors.[26] At pH 4.5, the range of linear responses of the *T.v.*-CDH-modified graphite electrode are identified from Figure 2A, and they are calculated to be up to 75 μM for cellobiose and up to 250 μM for lactose, with a corresponding sensitivity for cellobiose detection of 5 μA/mM (equal to 68.5 μA/cm^2·mM) and sensitivity for lactose detection of about 1.3 μA/mM (equal to 17.8 μA/cm^2·mM; area of electrode of 0.073 cm^2).

As a comparison, the sensitivities for cellobiose of other biosensors based on *P.c.*-CDH reported in the literature are 70 μA/cm^2·mM[25] or 4 μM/cm^2·mM.[31] Even though the substrate sensitivities of *T.v.*-CDH-modified electrodes seem to be similar to the ones modified with *P.c.*-CDH, an important difference must be pointed out. These other CDH biosensors are based on immobilization of *P.c.*-CDH with an $Os^{2+/3+}$ complex containing polymer that ensures a much higher amount of loaded enzyme in comparison with the amount of *T.v.*-CDH that can be adsorbed in a monolayer at the surface of a graphite electrode. Additionally, the redox conductive polymer may induce diffusion limitations of the substrate and product; and moreover it can open up the sensing system for interfering substances (especially at the reported potential of +450 mV vs Ag|AgCl[25]), as the $Os^{2+/3+}$-based mediator may catalyze their conversion.

From the above considerations, the use of *T.v.*-CDH-modified electrodes for the development of third-generation biosensors (using DET) for cellobiose and lactose seemed as a straightforward step in our research direction. The first analytical application of *T.v.*-CDH was demonstrated for the detection of lactose in products from the diary industry.[26] Due to its remarkable sensitivity for lactose detection, the *T.v.*-CDH biosensor has the potential application for sensitive lactose determination of those diary products, today commercialized as "lactose free", and dedicated to lactose-intolerant persons.

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Supporting Information Available: Supplimentary tables and figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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