Appl Biochem Biotechnol (2011) 163:404–414
DOI 10.1007/s12010-010-9048-3

Probing Reactivity of PQQ-Dependent Carbohydrate Dehydrogenases Using Artificial Electron Acceptor

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Received: 12 February 2010 / Accepted: 19 July 2010 /
Published online: 9 October 2010

Abstract The kinetic parameters of carbohydrate oxidation catalyzed by *Acinetobacter calcoaceticus* pyrroloquinoline quinone (PQQ)-dependent glucose dehydrogenase (GDH) and *Escherichia coli* PQQ-dependent aldose sugar dehydrogenase (ASDH) were determined using various electron acceptors. The radical cations of organic compounds and 2,6-dichlorophenolindophenol are the most reactive with both enzymes in presence of glucose. The reactivity of dioxygen with ASDH is low; the bimolecular constant k_{ox}=660 M^{-1} s^{-1}, while GDH reactivity with dioxygen is even less. The radical cation of 3-(10*H*-phenoxazin-10-yl)propionic acid was used as electron acceptor for reduced enzyme in the study of dehydrogenases carbohydrates specificity. Mono- and disaccharide reactivity with GDH is higher than the reactivity of oligosaccharides. For ASDH, the reactivity increased with the carbohydrate monomer number increase. The specificity of quinoproteins was compared with specificity of flavoprotein *Microdochium nivale* carbohydrate oxidase due to potential enzymes application for lactose oxidation.

Keywords Carbohydrate · Lactose · PQQ · Glucose dehydrogenase · Electron acceptor

Introduction

Quinoprotein oxidoreductases contain as a cofactor *o*-quinone derivative produced from an amino acid. The most typical *o*-quinone cofactor is pyrroloquinoline quinone (PQQ). Methanol dehydrogenase and glucose dehydrogenase are classic examples of quinoproteins containing PQQ as a cofactor [1]. Quinoproteins can be distinguished by their localization: soluble or membrane bound.

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Glucose dehydrogenase has been found both as soluble and membrane bound [1, 2]. Soluble PQQ-dependent glucose dehydrogenase (GDH), originating from *Acinetobacter calcoaceticus*, is a homodimeric enzyme containing one PQQ and three Ca^{2+} ions per monomer [3]. The enzyme has broad substrate specificity. GDH catalyzes the oxidation of monosaccharides such as glucose, xylose, and galactose, as well as disaccharides such as lactose and melibiose. The products of carbohydrates oxidation are corresponding lactones [2, 4]. GDH acts according to the ping-pong mechanism in which substrate inhibition by glucose and negative cooperativity effect were demonstrated [4]. The structure of the enzyme has been resolved, and the catalytic mechanism of the reductive half-cycle has been elucidated [5, 6].

Very recently, a soluble aldose sugar dehydrogenase (ASDH) was purified from *Escherichia coli*, and three-dimensional structure was determined [7]. The cofactor (PQQ) is bound in a large cleft of protein surface and is uniquely accessible to the water in comparison to other PQQ enzymes. The ASDH has a low (18%) identity with the *A. calcoaceticus*-soluble glucose dehydrogenase, but many amino acid residues involved in binding of the PQQ cofactor are conserved in both enzymes. ASDH has a low affinity for glucose and broad substrate specificity for aldose carbohydrates. The catalytic and structural properties of ASDH propose that this enzyme belongs to a new subgroup of PQQ-dependent soluble dehydrogenases that is distinct from the *A. calcoaceticus*-soluble dehydrogenase subgroup [7].

Practical application of carbohydrate dehydrogenases covers bioanalysis and carbohydrates modifications [8]. During biocatalytical conversion, carbohydrates are oxidized by oxidized enzyme following the reduced enzyme oxidation by electron acceptors. The high reduced enzyme oxidation rate is an important step for effective enzyme turnover. PQQ-dependent carbohydrate oxidases (COs) typically react slowly with dioxygen, and different artificial electron acceptors are used [4, 9–11].

The aim of the present work was dual: (a) to determine the reactivity of PQQ-dependent carbohydrate dehydrogenases with various electron acceptors and (b) to determine specificity of mono-, di-, and oligosaccharide oxidation using artificial electron acceptors. As PQQ dehydrogenases, glucose dehydrogenase from *A. calcoaceticus* (GDH) and aldose sugar dehydrogenase from *E. coli* (ASDH) were used.

Materials and Methods

Soluble glucose dehydrogenase was purified from *A. calcoaceticus* LMD 79.41 as described [4]. The final product was the enzyme solution with specific activity ~1,800 U/mg of protein. The recombinant soluble aldose sugar dehydrogenase encoded by *yliI* gene was purified from *E. coli*. The pET M-11*yliI* construct [7] was transformed into *E. coli* BL21 (DE3) cells. Single colonies were used to inoculate 5 ml of brain heart infusion (BHI) medium containing kanamycin at 37 °C for 16 h. This culture was used to inoculate 500 ml of BHI medium containing kanamycin. Cultures were grown until late log phase ($A_{600\ nm}$ approximately 0.8) and then cooled to 25 °C prior to the induction of protein expression with 0.5 mM isopropyl-β-D-thiogalactopyranoside. The cells were maintained at 25 °C for 5 h and then harvested by centrifugation (3,000×*g*, 20 min, 4 °C). Purification of ASDH and reconstitution with PQQ was carried out as described [7]. The final product was ASDH solution with specific activity 1.8 U/mg of protein. Molar concentration of enzymes was determined by Lowry method [12] using molecular mass of GDH 100 kDa [9] and of ASDH 39 kDa [7]. The recombinant *Coprinus cinereus* peroxidase (rCiP) and *Aspergillus*

niger catalase were received from Novo Nordisk A/S (Denmark) and used without further purification. Concentration of rCiP and catalase was determined spectrophotometrically at the wavelength of 405 nm using the molar extinction coefficient of 1.08×10^5 M^{-1} cm^{-1} [13].

Glucose, maltose, lactose, cellobiose, maltotriose, and maltotetraose were from "Sigma"; cellotriose and cellotetraose were from "Merck". PQQ, galactose, kanamycin, and isopropyl-β-D-thiogalactopyranoside were from "Fluka". Brain heart infusion medium was purchased from "Oxoid". Solutions of the carbohydrates were made in triple distilled water and allowed to mutarotate 1 day before use. All the concentrations of carbohydrates and calculated kinetic parameters are expressed in the terms of total amount of α and β anomers. The solution of hydrogen peroxide was prepared from 30% hydrogen peroxide (Carl Roth GmbH + Co), and the concentration was determined spectrophotometrically by using extinction coefficient of 39.4 M^{-1} cm^{-1} at 240 nm [14]. Buffer reagents and other chemicals were of analytical grade.

3-(10*H*-Phenoxazin-10-yl)propionic acid (PPA), 3-(10*H*-phenoxazin-10-yl)-propane-1-sulfonic acid (PPSA), and 10-methyl-10*H*-phenoxazine (MPX) were synthesized as described in [15]. 1-(*N*,*N*-Dimethylamine)-4-(4-morpholine)benzene (AMB) was synthesized as described in [16]. 2,6-Dichlorophenolindophenol sodium salt dihydrate (DCPIP) was from Sigma. Radical cation of 5,10-dimethyl-5,10-dihydro-phenazine ($DMDHP^{+\bullet}$) was synthesized by oxidizing DMDHP with hydrogen peroxide in ethanol containing hydrochlorous acid. The radical cations of the phenoxazine derivatives and AMB were prepared by adding 20 μl rCiP (1 μM) and 4×25 μl of hydrogen peroxide (20 mM) to 2 ml (0.5 mM) of the compound in the buffer solution. The reaction was terminated after 5 min by adding 20 μl of catalase. Final concentration of catalase was 100 nM. The solutions of the radical cations of PPA, PPSA, MPX, and AMB were prepared freshly before measurements and were kept in ice during the measurements.

The reduction of radical cations and DCPIP was monitored spectrophotometrically. The kinetic curves were recorded at the wavelength of maximum of absorbance of radical cation. The concentrations of the electron acceptors were calculated using extinction coefficients of 16 mM^{-1} cm^{-1} at 530 nm for $PPA^{+\bullet}$, $PPSA^{+\bullet}$, and $MPX^{+\bullet}$ [15, 17, 18], 7.4 mM^{-1} cm^{-1} at 462 nm for $DMDHP^{+\bullet}$ [19], 9.8 mM^{-1} cm^{-1} at 604 nm for $AMB^{+\bullet}$ [16], and 19.6 mM^{-1} cm^{-1} at 600 nm for DCPIP.

The kinetics of the dioxygen consumption was measured by using a homemade computer-assisted membrane oxygen electrode and 1.1 ml cell thermostated at 25 °C. The experiments were conducted in 5 mM Tris-HCl buffer solution, pH 8.0, containing 1 mM $CaCl_2$ and 0.2 M glucose. The concentration of dioxygen in air-saturated buffer solution at 25 °C was assumed to be 0.25 mM [20].

Calculations

The initial reaction rate of radical cations reduction was calculated as a slope of absorbance change during 10–30 s. The exponential curve of DCPIP absorbance decrease was approximated by first-order reaction kinetics, and initial reaction rate was calculated as $C_0\times k$, where C_0 is initial concentration of the substrate and k is first-order reaction constant. The dependence of dioxygen reduction rate on dioxygen concentration was obtained by digital differentiation of the kinetic curves of dioxygen consumption at fixed dioxygen concentration. In calculations, dioxygen and DCPIP were assumed as two electron acceptors. The radical cations are single electron acceptors, and for this reason, the rate of their reduction was divided by 2 to calculate the rate of substrate oxidation.

The analysis of the dependence of the initial rate on acceptor (A) or substrate (S) concentrations was performed by applying the ping-pong scheme [3, 4, 19]:

$$E_{ox} + S \underset{k_{-1}}{\overset{k_1}{\leftrightarrow}} E_{ox} - S \quad \overset{k_2}{\rightarrow} E_{red} + P \tag{1}$$

$$E_{red} + A_{ox} \overset{k_3}{\rightarrow} E_{ox} + A_{red} \tag{2}$$

where E_{ox}, E_{red}, $E_{ox} - S$, S, P, A_{ox}, and A_{red} denote the oxidized and reduced forms of enzyme, the enzyme–substrate complex, the substrate, the product, and the oxidized and reduced forms of electron acceptor, respectively. Following this scheme, the dependence of the initial reaction rate on substrates' and enzyme concentrations was expressed:

$$[E]_t/V_0 = (1/k_{cat}) + \left(1/\left(k_{red} \times [S]_0\right)\right) + \left(1/\left(k_{ox} \times [A]_0\right)\right) \tag{3}$$

where $k_{cat}=k_2$, $k_{red} = k_1 \times k_2/(k_{-1} + k_2)$, and $k_{ox}=k_3$ are apparent catalytic, reductive, and oxidative constants, respectively, and $[A]_0$ and $[S]_0$ are initial acceptor and carbohydrate concentrations, respectively. $[E]_t$ is a total enzyme concentration.

At large excess of substrate (carbohydrate), the dependence of the reaction initial rate on substrate, enzyme, and electron acceptor concentrations could be simplified:

$$V_0 = k_{cat} \times k_{ox} \times [E]_t \times [A]_0/\left(k_{ox} \times [A]_0 + k_{cat}\right) \tag{4}$$

At a low electron acceptor concentration, when $k_{cat} > k_{ox} \times [A]_0$, the initial rate is linearly proportional to the electron acceptor concentration:

$$V_0 = k_{ox} \times [E]_t \times [A]_0 \tag{5}$$

At a high (saturated) concentration of electron acceptor, when $k_{ox} \times [A]_0 > k_{red} \times [S]_0$, the Eq. 3 could be simplified:

$$V_0 = k_{cat} \times k_{red} \times [E]_t \times [S]_0/\left(k_{red} \times [S]_0 + k_{cat}\right) \tag{6}$$

At a low substrate concentration, when $k_{cat} > k_{red} \times [S]_0$, the initial rate is linearly proportional to the carbohydrate concentration:

$$V_0 = k_{red} \times [E]_t \times [S]_0 \tag{7}$$

The dependence of initial rate on substrate (carbohydrate) or electron acceptor (A) concentration was fitted by Michaelis–Menten (Eqs. 4 and 6) or linear anamorphosis (Eqs. 5 and 7), and calculated parameters were used to evaluate k_{ox} and k_{red}:

$$k_{ox} = V_{max}/\left(K_{M(A)} \times [E]_t\right) \tag{8}$$

$$k_{ox} = \text{slope}/[E]_t \tag{9}$$

and

$$k_{red} = V_{max}/\left(K_{M(S)} \times [E]_t\right) \tag{10}$$

$$k_{red} = \text{slope}/[E]_t \tag{11}$$

where $K_{M(A)} = k_{cat}/k_{ox}$ and $K_{M(S)} = k_{cat}/k_{red}$ are apparent Michaelis–Menten constants for electron acceptor and carbohydrate, respectively.

Results and Discussion

Kinetics of Dehydrogenases with Artificial Electron Acceptors

It was demonstrated that dioxygen reacts slowly with PQQ-dependent dehydrogenases [9–11]. GDH at concentration of 5.5 μM and in presence of 0.1 M of glucose did not show significant dioxygen consumption. The estimated oxidation constant was less that 5 M^{-1} s^{-1}. ASDH, however, catalyzes glucose oxidation by dioxygen at higher rate (Fig. 1). The initial reaction rate is directly proportional to enzyme concentration (Fig. 1, insert). The dependence of reaction rate on dioxygen concentration at various ASDH concentrations was also linear (Fig. 2). The calculated k_{ox} value was equal to $(6.6\pm0.6)\times 10^2$ M^{-1} s^{-1}. This value is significantly less in comparison to flavin oxidases; it is 4.5×10^5 M^{-1} s^{-1} for glucose oxidase from *A. niger* [21] and 1.7×10^5 M^{-1} s^{-1} for carbohydrate oxidase *Microdochium nivale* [19]. Low oxidation constant with dioxygen does not allow practical use of dioxygen as oxidizer even for ASDH catalyzed process.

In trying to find effective electron acceptors, radical cations of *N*-substituted phenoxazines, *N*,*N*-substituted phenazine, and *N*,*N*-substituted *p*-phenylendiamine were used. These radical cations are stable in water solution [22]. The electrochemical conversion of the radical cations is fast, and for this reason, electrochemical method of

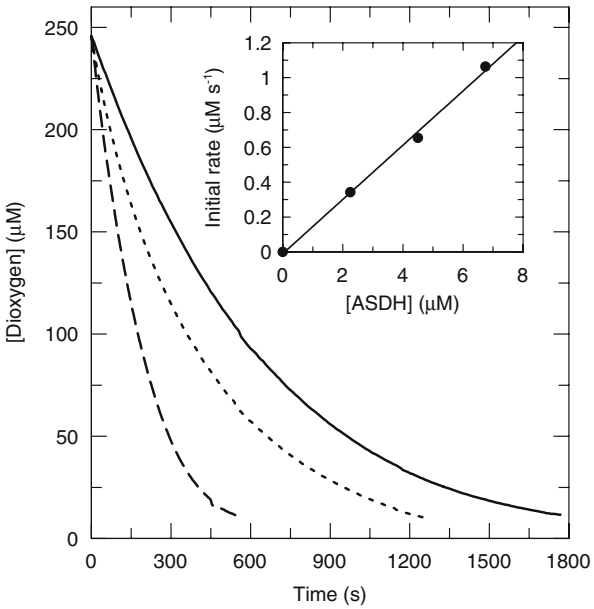

Fig. 1 Dioxygen concentration change at various ASDH amounts in presence of 0.2 M glucose at 25 °C. *Inserted graph* shows the dependence of the initial rate of dioxygen consumption on ASDH concentration. The *curve drawn though the points* is a linear approximation

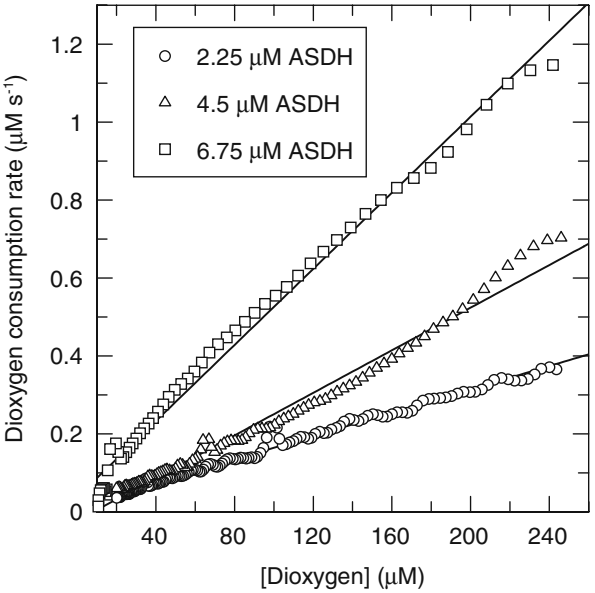

Fig. 2 The dependence of reaction rate on dioxygen concentration at various ASDH concentrations. The *curves drawn though the points* are linear approximations. Conditions are the same as in Fig. 1

the acceptors regeneration can be applied [15]. High reactivity of some radical cations with PQQ-dependent dehydrogenases was demonstrated in previous preliminary investigation [23].

The reduction of radical cations proceeded at GDH concentration as low as 1 nM (Fig. 3). Oxidative constant calculated using Eq. 9 was $(2.2\pm0.4)\times10^7$ M^{-1} s^{-1} for radical cation of PPA (Table 1). The constants for other acceptors are even larger. They approached diffusion-limited values for enzyme catalysis [24]. The reactivity does not correlate with redox potential of acceptors since E'_0 is 0.634, 0.339, and 0.390 V for PPA, DMDHP, and AMB, respectively [15, 16, 25]. This in addition indicates possible diffusion limitation of radical cations reactivity.

The reactivity of radical cations was compared with DCPIP, which was used in original investigations [9]. The calculated constant of GDH with DCPIP is lower than that of radical cations (Table 1).

Reduced ASDH reacts with radical cations at 20–250 nM concentration. The calculated constants varied between 1.0×10^6 and 2.3×10^7 M^{-1} s^{-1} (Table 1). The constant values do not correlate with redox potential of acceptor.

The kinetics of DCPIP reduction was performed at various glucose concentrations. The analysis of initial rate dependence on DCPIP concentration at various glucose concentrations was performed applying the ping-pong scheme of enzyme action (Eqs. 1 and 2) and the expression of the initial rate dependence on enzyme, substrate, and electron acceptor concentration (Eq. 3). According to the Eq. 3, the dependence of reverse rate on reverse DCPIP concentration is linear, and the change of glucose concentration generates parallel lines. Experimentally obtained dependences of reverse rate on reverse DCPIP concentration were near linear and parallel at different glucose concentrations that confirm the ping-pong scheme of ASDH action. The reactivity of DCPIP was lower than the reactivity of radical cations, as in the case of GDH.

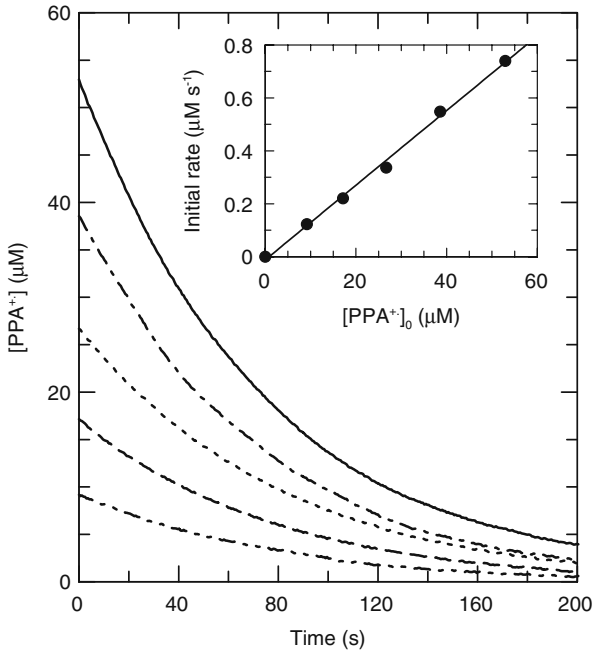

Fig. 3 Kinetic curves of $PPA^{+\bullet}$ concentration change at 0.4 nM GDH and 20 mM glucose. The *insert graph* shows the dependence of initial reaction rate on $PPA^{+\bullet}$ concentration; the *curve drawn through the points* is an approximation of the data by linear equation

Table 1 Oxidation constants of GDH and ASDH reaction with radical cations and DCPIP at 25 °C

Electron acceptor	Enzyme	Buffer solution	pH	$C_{glucose}$, M	k_{ox}, $M^{-1}s^{-1}$
$PPA^{+\bullet}$	GDH	Phosphate	7.2	0.020	$(3.8\pm0.1)\times10^7$
$PPA^{+\bullet}$	GDH	Imidazol	7.0	0.020	$(2.2\pm0.4)\times10^7$
$AMB^{+\bullet}$	GDH	Imidazol	7.0	0.020	$(4.9\pm1.0)\times10^7$
$DMDHP^{+\bullet}$	GDH	Imidazol	7.0	0.020	$(4.8\pm0.4)\times10^7$
DCPIP	GDH	Imidazol	7.0	0.020	$(8.6\pm2.4)\times10^6$
$PPA^{+\bullet}$	ASDH	Phosphate	7.2	0.11	$(2.3\pm1.1)\times10^6$
$PPA^{+\bullet}$	ASDH	TRIS	8.0	0.11	$(1.0\pm0.2)\times10^6$
$MPX^{+\bullet}$	ASDH	TRIS	8.0	0.20	$(5.2\pm3.3)\times10^6$
$PPSA^{+\bullet}$	ASDH	TRIS	8.0	0.22	$(2.3\pm0.7)\times10^7$
$AMB^{+\bullet}$	ASDH	TRIS	8.0	0.20	$(1.6\pm2.7)\times10^7$
$DMDHP^{+\bullet}$	ASDH	TRIS	8.0	0.17	$(1.0\pm0.2)\times10^6$
DCPIP	ASDH	TRIS	8.0	0.032	$(3.7\pm1.3)\times10^5$
DCPIP	ASDH	TRIS	8.0	0.064	$(3.3\pm1.5)\times10^5$
DCPIP	ASDH	TRIS	8.0	0.13	$(3.2\pm0.9)\times10^5$
DCPIP	ASDH	TRIS	8.0	0.23	$(4.4\pm1.4)\times10^5$

Specificity of Dehydrogenases

To evaluate specificity of dehydrogenases, radical cation of PPA was chosen. The oxidation of carbohydrates was performed at constant electron acceptor concentration at pH 7.2. The calculated k_{red} was used as a parameter related to specificity.

The kinetic curves of the radical cation concentration decrease were almost linear up to larger conversion (Figs. 3 and 4). The linear dependence of initial rate on substrate (carbohydrate) concentration indicates that limiting reaction is substrate oxidation (Fig. 3). In this case, k_{red} was calculated using Eq. 11. The saturating character of the dependence of the initial rate on substrate concentration indicated unsatisfactory concentration of acceptor at high substrate concentration. However, the slope calculated as $V_{max}/(K_{M(S)})$ can be used for k_{red} evaluation (Eq. 10).

In the case of GDH, the calculated bimolecular constants of carbohydrates oxidation span a range 4.6×10^4–6.9×10^5 M^{-1} s^{-1} (Table 2). The k_{red} shows that GDH shows quite broad specificity and reacts effectively with monosaccharides and disaccharides, such as maltose or lactose. The reactivity of substrates depends on the length of the carbohydrate chain. Mono- and disaccharides are more reactive in comparison to oligosaccharides. The determined specificity is typical for the soluble PQQ-dependent carbohydrate dehydrogenases [4] (Fig. 5).

The reactivity ASDH toward carbohydrates is about 4 orders of magnitude less in comparison to GDH (Table 2). A tendency of reactivity increase was indicated for oligosaccharides. The larger ASDH reactivity toward oligosaccharides can be related to the structure of the open active center [7].

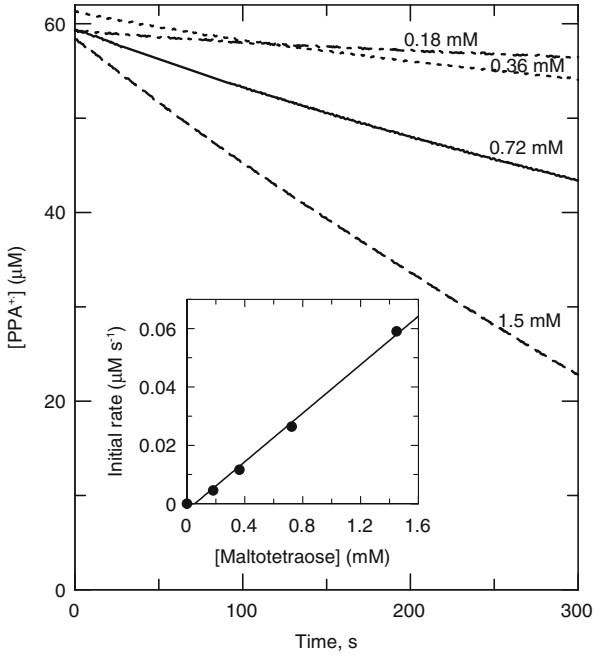

Fig. 4 GDH catalyzed reduction of $PPA^{+\bullet}$ in presence of maltotetraose. The concentrations of maltotetraose are shown on the *curves*. The concentration of GDH was 1.0 nM. The *insert graph* shows the dependence of the initial reaction rate on maltotetraose concentration

Table 2 The reactivity of GDH, ASDH, and CO at 25 °C

Carbohydrate	GDH pH 7.2 k_{red} (M^{-1} s^{-1})	ASDH pH 7.2 k_{red} (M^{-1} s^{-1})	ASDH pH 8.0 k_{red} (M^{-1} s^{-1})	CO pH 7.2 k_{red} (M^{-1} s^{-1})
Glucose	$(6.9\pm1.2)\times10^5$	1.4±0.3	11±3	$(3.61\pm0.04)\times10^2$
Galactose	$(9.2\pm1.0)\times10^4$	4.6±0.5	25±7	$(6.8\pm0.3)\times10^1$
Lactose	$(1.4\pm0.9)\times10^5$	9.3 ±2.7	28±7	$(3.0\pm0.5)\times10^5$
Cellobiose	$(4.9\pm0.4)\times10^5$	6.5±1.1	–	$(5.6\pm0.4)\times10^5$[a]
Cellotriose	$(3.5\pm0.4)\times10^5$	48±3	–	$(1.2\pm0.2)\times10^5$[a]
Cellotetraose	$(2.3\pm0.5)\times10^5$	75±3	–	$(8.5\pm1.6)\times10^4$[a]
Maltose	$(3.8\pm1.1)\times10^5$	6.7±1.3	–	$(9.7\pm0.9)\times10^2$[a]
Maltotriose	$(1.4\pm0.5)\times10^5$	20±2	–	$(1.3\pm0.6)\times10^3$[a]
Maltotetraose	$(4.6\pm0.2)\times10^4$	23±7	–	$(2.2\pm0.6)\times10^3$[a]

[a] Data from [26]

The low ASDH reactivity can be associated with non-optimal pH. Southall et al. [7] demonstrated that the activity of ASDH toward the substrates increases at pH 8.75. For this reason, reductive constant of ASDH for three substrates—glucose, galactose, and lactose—was measured at pH 8.0. The obtained values of k_{red} at pH 8.0 are higher than at pH 7.2, but the relative specificity among these three substrates is the same at pH 7.2 and 8.0.

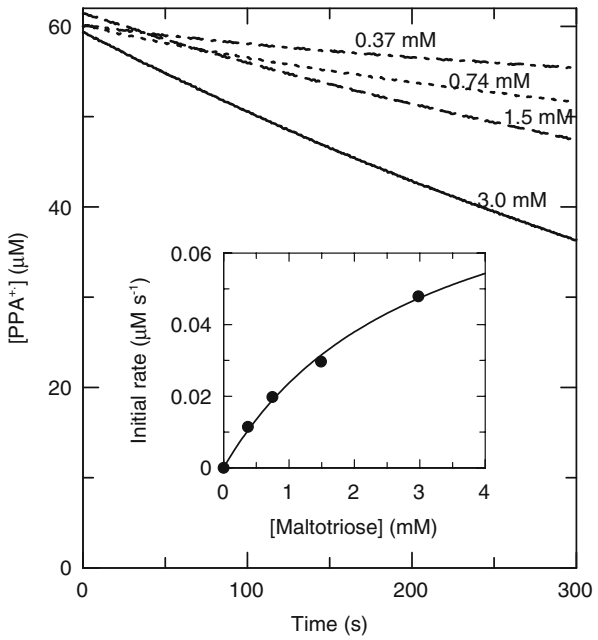

Fig. 5 GDH catalyzed reduction of $PPA^{+\bullet}$ in presence of maltotriose. The concentrations of maltotriose are shown on the *curves*. The concentration of GDH was 0.2 nM. The *insert graph* shows the dependence of the initial reaction rate on maltotriose concentration

Concluding Remarks

In this study, we have characterized the reactivity of two PQQ-dependent carbohydrate dehydrogenases toward various substrates and electron acceptors. Both enzymes show the wide substrate specificity acting on mono-, di-, and oligosaccharides. Moreover, various electron mediator systems might be applied to regenerate these dehydrogenases. One of possible PQQ carbohydrate dehydrogenase applications is lactobionic acid (LA) synthesis from lactose. LA is an excellent biodegradable chelator and can be used for preparation of detergents. The use of carbohydrate oxidase from *M. nivale* for lactose oxidation was suggested very recently [27]. The reactivity of GDH toward lactose is similar in comparison to CO (Table 2). However, the specificity of both enzymes is different. GDH catalyzes the oxidation of di- and oligosaccharides with α-glycoside and β-glycoside bonds at a similar rate. In contrast, CO shows much higher reactivity toward carbohydrates containing β-glycoside bonds. Given a suitable electron acceptor, GDH can be used for LA production from lactose. The constant of reactivity of ASDH with lactose is very low, and the use of this enzyme for lactobionic acid might need large amount of protein. However, the wide substrate specificity of ASDH means possibilities to use the enzyme in combination with an appropriate hydrolase for modification of polysaccharides, in addition to development of biosensors.

Acknowledgments The research was supported by Lithuanian State Science and Studies Foundation, grant No N-09/2007. The authors thank Dr. S.M. Southall for the pET M-11*yliI* construct and Dr. R. Vidziunaite and M. Dagys for assistance in measurements.

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