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An unexpectedly high reactivity of carbohydrate oxidase to permanganate

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The oxidation of cellobiose with permanganate catalyzed with recombinant *Microdochium nivale* carbohydrate oxidase was studied at pH 5.5–8.0 and 25 °C. An apparent bimolecular constant for carbohydrate oxidase with permanganate reached a value of 15 $\mu M^{-1}s^{-1}$ at pH 6.0. The dependence of the bimolecular constant on the ionic strength of the solution revealed the negatively charged group of carbohydrate oxidase to control the permanganate reaction.

Analysis of full kinetic curves of permanganate reduction showed an unexpected decrease in reduction rate during the reaction, which was explained by product, *i.e.* manganate, disproportionation. The model fitted the experimental data, and the calculated manganate disproportionation rate was 40 $M^{-1}s^{-1}$ and 9.8 $M^{-1}s^{-1}$ at pH 6.0 and 7.2, respectively.

The reactivity of carbohydrate oxidase with permanganate was almost 74 times higher than the reactivity of *Aspergillus niger* glucose oxidase at pH 7.2, indicating a different surrounding of reduced flavin intermediates in the oxidases.

Key words: carbohydrate oxidase, *Microdochium nivale*, kinetics, potassium permanganate

INTRODUCTION

Flavoprotein oxidases containing flavin as a cofactor may catalyze the oxidation of carbohydrates at the 1st or 2nd position [1–8]. During turnover both types of oxidases produce reduced flavin, which reacts with different electron acceptors [1–8]. Flavin containing carbohydrate oxidase from *Microdochium nivale* (rMnO) oxidized the β-form of D-glucose and other aldoses reacting with them in the 1-position; the product of D-glucose oxidation was D-gluconic acid [9–11]. Compared to *Aspergillus niger* glucose oxidase (GO), the reactivity of rMnO at pH 7.0 was significantly lower using oxygen or two-electron acceptors, but it showed a much higher activity towards single electron acceptors. In an attempt to find new electron acceptors, we unexpectedly found that potassium permanganate may act as an effective rMnO oxidizer. Though this compound is known as a strong chemical oxidizer [12, 13], to the best of our knowledge it has not been used in oxidase reactions.

MATERIALS AND METHODS

The recombinant carbohydrate oxidase from *Microdochium nivale* (rMnO), other enzymes and materials were used as in [10, 11]. The kinetic measurements were performed as described in [11].

The analysis of the rate dependence on permanganate concentration was based on a ping-pong scheme for enzyme action [11]:

$$V = V_{max} \cdot [M]_0 \cdot [S]_0 / ([M]_0 \cdot [S]_0 + K_m \cdot [S]_0 + K_s \cdot [M]_0), \quad (1)$$

where V_{max}, K_m and K_S are the maximum rate and apparent Michaelis–Menten constant for permanganate and carbohydrate (glucose or cellobiose), $[M]_0$ and $[S]_0$ are the initial permanganate and carbohydrate concentrations.

The dependence of the rate on permanganate concentration obtained at excess of carbohydrate concentration was fitted by the monosubstrate Michaelis–Menten equation (eq. 2).

$$V = V_{max} \cdot [M]_0 / (K_m + [M]_0). \quad (2)$$

The integrated form of the monosubstrate Michaelis–Menten equation was expressed as

$$t = K_m/V_{max} \cdot \ln([M]_0 / [M]_t) + (1/V_{max}) \cdot ([M]_0 - [M]_t), \quad (3)$$

where $[M]_t$ is the permanganate concentration during the reaction.

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The calculated V_{max} and K_m values were used for the calculations of the apparent rate constants (k_{cat} and k_{ox}):

$$k_{cat} = V_{max} / [E]_t, \quad (4)$$

$$k_{ox} = k_{cat} / K_m, \quad (5)$$

where $[E]_t$ is the total enzyme concentration.

The modeling of the full kinetic curves of permanganate reduction was performed digitally. The scheme included permanganate ion (Mn^{7+}) reduction (eq. 6) by the following Michaelis–Menten scheme and the two reactions associated with manganate (Mn^{6+}) disproportionation (equations 7 and 8):

$$Mn^{7+} \rightarrow Mn^{6+} \qquad V \quad (6)$$

$$Mn^{6+} + Mn^{6+} \rightarrow Mn^{5+} + Mn^{7+} \qquad k_1 \quad (7)$$

$$Mn^{6+} + Mn^{5+} \rightarrow Mn^{4+} + Mn^{7+} \qquad k_2 \quad (8)$$

RESULTS AND DISCUSSION

Initial reaction rate

At pH 7.2 and in the presence of 1.8 mM of cellobiose and 18 nM of rMnO the violet color of the solution bleached during 10 min. The data presented in Fig. 1 show that the permanganate reduction rate changed only little at a cellobiose concentration of 1.7 mM or 4.3 mM, indicating that the K_s at pH 7.2 was much less that 1.7 mM. The cellobiose concentration of 2 mM was used to determine the permanganate reduction rate, and the nonenzymatic reduction of permanganate was negligible at this carbohydrate concentration.

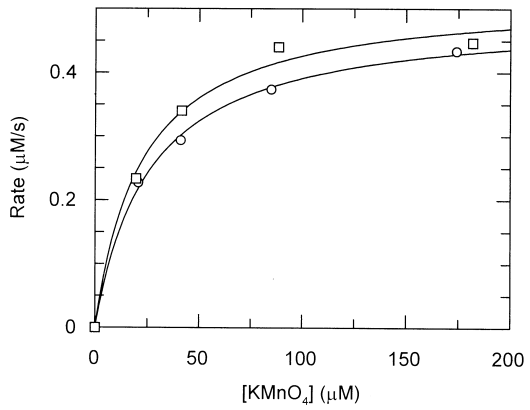

Fig. 1. Dependence of initial reaction rate on permanganate concentration. Cellobiose 4.3 mM (○), 1.7 mM (□), 50 mM phosphate buffer solution, pH 7.2, aerobic conditions, 0.1 μM catalase, 10 nM rMnO. The curves were a fit of the data by monosubstrate Michaelis–Menten equation

Table 1. **Catalytic parameters of rMnO-catalyzed permanganate reduction at different pH. 50 mM phosphate buffer solution, 2 mM cellobiose, 25 °C**

pH	$KMnO_4$, μM	k_{cat}, s^{-1}	K_m, μM	k_{ox}, $\mu M^{-1}s^{-1}$
5.5	20–241	24 ± 1.6	14.7 ± 1.0	1.6 ± 0.2
5.8	15–150	43.7 ± 5.1	5.4 ± 0.6	8.1 ± 1.9
6.0	16–145	44.5 ± 1.8	2.9 ± 0.1	15 ± 1.2
6.5	15–144	51.4 ± 5.6	4.9 ± 0.5	10.5 ± 2.3
6.8	21–145	47.2 ± 5.4	5.0 ± 0.6	9.5 ± 2.2
7.0	34–244	51.9 ± 3.8	7.4 ± 0.5	6.9 ± 1.0
7.2	18–162	62.9 ± 2.7	16.8 ± 0.7	3.7 ± 0.3
7.5	17–148	56.9 ± 2.9	18.1 ± 0.9	3.1 ± 0.3
8.0	26–223	47.3 ± 5.4	17.3 ± 2.0	2.7 ± 0.6

The reactivity of rMnO with permanganate was investigated at pH 5.5–8.0 (Table). The calculated catalytic constant values correlate well with those calculated earlier when oxygen was used as an electron acceptor [11]. This is consistent with a ping-pong scheme of permanganate reduction.

The maximum value of the bimolecular constant of permanganate action (k_{cat} / K_m) was 15 $\mu M^{-1}s^{-1}$ at pH 6.0. The k_{ox} values decreased at pH lower than 6.0. At pH less than 6 the reaction was associated with a 3-electron permanganate reduction and manganese dioxide formation (equations 6, 7, 8). Therefore, the measured rate was slower and the calculated constant decreased. The decrease of the constant at pH higher than 6.0 could be attributed to the pH dependence of rMnO-catalyzed permanganate reduction (equation 6). The calculated apparent pK_a value of this transition was 6.7 ± 0.2.

Investigations of the dependence of the rMnO-catalyzed cellobiose oxidation rate on phosphate buffer concentration, showed that V_{max} (k_{cat}) and k_{ox} increased when the buffer concentration increased in the range 5–200 mM. To test whether this was due to a specific phosphate action, a set of experiments was carried out where the ionic strength of the solution was changed by adding sodium sulfate. The expression of linear dependence of $\log(k_{ox})$ on $\mu^{1/2}$ was $\log(k_{ox}) = (0.84 \pm 0.26) \cdot \mu^{1/2} + (0.21 \pm \pm 0.07)$ ($R = 0.7318$).

The dependence of the constant on the ionic strength of the solution can be approximated by the Debye-Hückel equation [14]:

$$\log (k) = \log (k_0) + 2 \cdot Z_A \cdot Z_B \cdot A \cdot \mu^{1/2}, \quad (7)$$

where k_0 is a constant at the zero ionic strength (μ), Z_A and Z_B are reagent charges, and the constant $A = 0.512$ at 25 °C in a water solution. There-

fore the slope of the linear dependence of log (k) on $\mu^{1/2}$ equals approximately to the multiplied charges of the reagents. The experimentally determined slope was 0.84. This means that the negatively charged group of the enzyme controls the permanganate reaction. The slope 0.96 was indicated for rMnO reaction with the cation radical of N,N-dimethylamino-(4-mopholinyl) benzene [11]. The cation radical possesses a positive charge, and the positive value of the slope indicates that a positively charged group of the enzyme controls the interaction with the cation radical.

The rMnO reactivity with permanganate was compared with that of GO, using glucose as the second substrate. At pH 7.2 the nonenzymatic permanganate reduction rate was low at the glucose concentration less than 2 mM. The K_s of GO-catalyzed permanganate reduction was 80 ± 3 μM in presence of 0.15 mM of permanganate. However, saturation of the reaction rate was established at a fixed glucose concentration of 2 mM and at a permanganate concentration larger than 0.15 mM. The maximum rate was 0.24 μM/s at GO 0.17 μM and the apparent K_m for permanganate was 28 ± 10 μM. The maximal rate in permanganate reaction was 17-fold less in comparison with k_{cat} [E] for GO in the presence of oxygen [3]. This indicated a complicated reduced GO interaction with permanganate and needed special investigation. Nevertheless, the response of the rate on permanganate concentration, established as k_{cat}/K_m, was $(5.0 \pm 2.1) \cdot 10^4$ M^{-1} s^{-1}. This value was almost 74 times less than the bimolecular constant of rMnO at the same pH. The results of rMnO reactivity with single electron acceptors, *i.e.* organic cation radicals [11], as well as with permanganate ion show that single reduced flavin intermediates in rMnO are more stabilized in comparison to *A. niger* glucose oxidase.

Kinetics of total permanganate reduction

Analysis of the full kinetic curves exposed an unexpected permanganate reduction rate decrease during the reaction. The permanganate concentration change calculated by using the integral Michaelis–Menten equation and the parameters acquired from the initial reaction rate was compared with the experimental values. The difference between the calculated and the experimental curves was larger at pH 6.0 than at pH 7.2.

To explain the unexpected decrease in the reaction rate, a disproportionation of the primary reaction product, *i.e.* manganate, was suggested (reactions 9, 10):

$$MnO_4^- + 1\ e^- \rightarrow MnO_4^{2-}, \quad (9)$$

$$3\ MnO_4^{2-} + 4\ H^+ \rightarrow 2\ MnO_4^- + MnO_2 + 2\ H_2O. \quad (10)$$

Since protons participated in this process, the reaction rate was faster in a more acid solution. To model this process, a scheme was used that included 2 bimolecular reactions of manganate disproportionation following permanganate and manganese dioxide formation (eq. 7, 8). The limiting step of disproportionation was one of the bimolecular processes.

A comparison of the experimental data with those calculated according to the model gave a satisfactory agreement of permanganate concentration change as well as reaction rate change. Figure 2 shows the experimental data and model calculation results at pH 6.0. The digital simulations gave manganate disproportionation rates of 40.0 ± ± 1.4 $M^{-1}s^{-1}$ and 9.8 ± 0.5 $M^{-1}s^{-1}$ at pH 6.0 and 7.2, respectively.

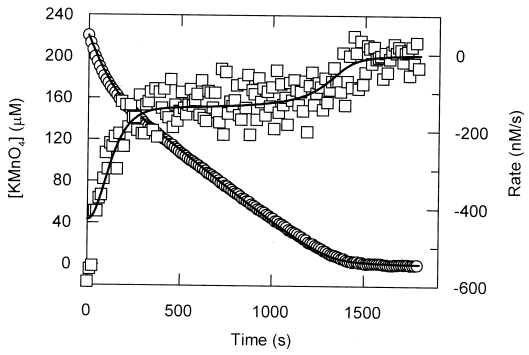

Fig. 2. Permanganate concentration (left hand *y* axis, o) and reduction rate change (right-hand *y* axis, □) during rMnO-catalyzed cellobiose oxidation at pH 6.0, rMnO 18 nM, cellobiose 1.8 mM, 0.1 μM catalase, aerobic conditions. Solid curves represent calculated values

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NELAUKTAI AUKŠTAS ANGLIAVANDENIŲ OKSIDAZĖS REAKTYVUMAS SU PERMANGANATU

S a n t r a u k a

Rekombinantinės *Microdochium nivale* angliavandenių oksidazės katalizuojama celobiozės oksidacija permanganatu buvo tiriama esant pH 5,5–8,0 ir 25 °C temperatūrai. Tariamosios permanganato ir angliavandenių oksidazės sąveikos bimolekulinės konstantos reikšmė siekia 15 $\mu M^{-1}s^{-1}$ esant pH 6,0. Bimolekulinės konstantos priklausomybė nuo tirpalo joninės jėgos parodė, kad angliavandenių oksidazės neigiamai įkrautos grupės valdo sąveiką su permanganatu.

Permanganato redukcijos visos kinetinės kreivės analizė atskleidė nelauktą permanganato redukcijos greičio sumažėjimą, kurį nulėmė manganato skilimas. Sukurtas modelis atitiko eksperimentinius duomenis, o apskaičiuotas manganato skilimo greitis buvo 40 $M^{-1}s^{-1}$ ir 9,8 $M^{-1}s^{-1}$ esant atitinkamiems pH dydžiams – 6,0 ir 7,2.

Angliavandenių oksidazės reaktyvumas su permanganatu esant pH 7,2 buvo maždaug 74 kartus aukštesnis nei *A. niger* gliukozooksidazės reaktyvumas, ir tai rodo skirtingą redukuoto flavininio kofaktoriaus apsuptį šiose oksidazėse.