The Effects of Oxyanions on the Activity of Oxalate Oxidase

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Abstract: Oxalate oxidase catalyzes the conversion of oxalic acid and molecular oxygen to carbon dioxide and hydrogen peroxide. Oxalate-degrading enzymes are of interest for various applications including clinical analysis of the levels of oxalic acid in blood and urine and control of oxalic acid in industrial processes, such as pulp and paper manufacture. In these applications, the presence of oxyanions other than oxalate may affect the enzyme activity. The inhibitory effect of selected oxyanions on oxalate oxidase from barley was investigated. Seven out of fourteen of the compounds studied inhibited oxalate oxidase at a concentration of 1 mM. Perchlorate, chlorate and chlorite were selected for more detailed studies. The results indicate that perchlorate, chlorate and chlorite cause mixed inhibition of oxalate oxidase and that the severity of the inhibition within the series increases with the oxidation state. The apparent K_M of the enzyme was 0.28 ± 0.05 mM.

Keywords: Oxalate oxidase, Oxalic acid, Inhibition, Oxyanions.

INTRODUCTION

In the presence of molecular oxygen, oxalate oxidase catalyzes the conversion of oxalic acid to carbon dioxide and hydrogen peroxide: $HOOC–COO^- + H^+ + O_2 \rightarrow 2\ CO_2 + H_2O_2$. Oxalate oxidase is widespread in nature and has been found in fungi, bacteria, and plants, such as barley and wheat [1]. It is a homohexamer and belongs to a large family of germin-like proteins that are termed cupins because of their conserved β-barrel fold [2]. The active site is found within the center of the β-barrel and contains a manganese ion [3].

Elevated levels of oxalic acid are associated with the formation of kidney stones consisting of calcium oxalate [1]. Oxalate oxidase is mainly used in clinical analysis, where it is used in assays for measurements of oxalate levels in blood and urine. Such assays are vital in the control of hyperoxaluria. Successful treatment of patients suffering from primary and secondary hyperoxaluria with the oxalate-degrading bacteria *Oxalobacter formigenes* has been reported [4], but enzymatic removal of oxalic acid offers an alternative. Calcium oxalate precipitation also causes problems in industrial processes, for example in the pulp and paper industry. Degradation of oxalic acid with oxalate oxidase could be a possible way to solve this problem [5].

Different mechanisms for degradation of oxalic acid with oxalate oxidase have been suggested [6,7]. Although some compounds are known to inhibit oxalate oxidase [8-15], the mechanism of inhibition has not been studied previously. Inhibitors affect the utilization of the enzyme in various applications. Furthermore, inhibitory studies are needed in order to find out more about the properties of the enzyme.

Oxalic acid is an oxyanion, and because of that it is of interest to investigate the effect of other oxyanions on the activity of oxalate oxidase. The effects of fourteen oxyanions and related compounds on oxalate oxidase from barley were studied and a series of chlorine-containing oxyanions was selected for kinetic analysis of the inhibitory effect.

MATERIALS AND METHODS

Screening of Potential Inhibitors

The following compounds were analyzed with respect to their effect on oxalate oxidase activity (reagent grade chemicals were used): sodium arsenate, sodium *m*-arsenite, sodium carbonate, sodium chlorate, sodium chlorite, DTPA (diethylenetriaminepentaacetic acid), EDTA (ethylenediaminetetraacetic acid), sodium molybdate, sodium nitrate, sodium nitrite, sodium perchlorate, potassium phosphate, sodium sulfite and sodium selenate.

The reaction temperature was 21°C. Absorbance measurements were done with a spectrophotometer (UV-1601PC, Shimadzu, Kyoto, Japan). The consumption of oxygen was measured using a Clark electrode (Hansatech Instruments, Kings Lynn, UK).

A coupled assay with ABTS was used to study arsenite, chlorate, nitrate, perchlorate, sulfite and selenate. The total volume of the assay was 1.0 ml. The assay mixture included the following components (final concentrations indicated): 50 mM succinate buffer (the pH of a solution of sodium succinate was adjusted to 3.8 using 5 M HCl); 1.0 mM ABTS (≥99.0%, Fluka, Buchs, Switzerland); 24 U/ml peroxidase (type VI from horseradish, Sigma-Aldrich, St Louis, MO, USA); 2.0 mM oxalic acid (pH was adjusted to 3.8 using 5 M NaOH); 0.1 mg/ml oxalate oxidase from barley seedlings

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(Sigma-Aldrich). The pH of the solutions of the tested compounds was adjusted to 3.8. Concentrations up to 20 mM were investigated. The absorbance at 414 nm was measured for 1.0 min.

Carbonate, chlorite, DTPA, EDTA, nitrite, arsenate, molybdate and phosphate all affected the peroxidase in the coupled assay and because of that they were instead studied using the Clark electrode. The total volume of the assay was 1.0 ml. The assay mixture included the same components as in the coupled assay described above, except that ABTS and horseradish peroxidase were omitted. The consumption of oxygen was measured for 3.0 min.

Kinetic Studies of Inhibitors

The inhibition of oxalate oxidase by chlorate, chlorite and perchlorate was studied using final concentrations resulting in 20–60% inhibition of the oxalate oxidase activity.

Chlorate and perchlorate were studied in assay mixtures that included the following components (final concentrations indicated): 50 mM succinate buffer (pH 3.8, adjusted with 5 M HCl); 1.0 mM ABTS, 24 U/ml horseradish peroxidase; oxalic acid solution (adjusted to pH 3.8 using 5 M NaOH) (17 different concentrations giving final concentrations of oxalic acid ranging between 0.10 and 2.0 mM); 0.1 mg/ml oxalate oxidase. The assay was also performed without any inhibitor present. The total volume of the assay mixture was 1.0 ml. The reaction time was 1.0-3.0 min depending on the velocity of the reaction. The V_0 values were calculated with reference to oxalate oxidase (assuming that the formation of two moles of ABTS radical cations is equivalent to the consumption of one mole of oxalic acid).

The Clark electrode was used for the study of chlorite. The assay mixtures included the following components (final concentrations indicated): 50 mM succinate buffer (pH adjusted to 3.8 with 5 M HCl); oxalic acid in 17 different concentrations (adjusted to pH 3.8 using 5 M NaOH) giving final concentrations ranging between 0.10 and 2.0 mM; 0.1 mg/ml oxalate oxidase. The assay was also performed without any inhibitor present. The total volume of the assay was 1.0 ml. The reaction time was 3.0 min.

RESULTS AND DISCUSSION

Screening of Potential Inhibitors

A screening of various oxyanions was performed to identify inhibitors of oxalate oxidase (Table **1**). Seven out of fourteen studied compounds showed an inhibitory effect on oxalate oxidase from barley at a concentration of 1 mM (Table **1**). Perchlorate and sulfite inhibited the enzyme completely at a concentration of 1 mM. Larsson *et al*. [12] reported that the enzyme had no remaining activity at a sulfite concentration of 1 mM which agrees with the result from this study. No information regarding the inhibitory effect of perchlorate has been reported earlier.

Chlorate and nitrite were strong inhibitors of oxalate oxidase. When the inhibitor concentration was 1 mM, the remaining activity was 8% (Table **1**). Larsson *et al*. [12] reported that 62% of the activity remained in the presence of 1 mM chlorate. The inhibitory effect observed in the present study was more severe, which could be due to the fact that different types of assays have been used.

Chlorite, molybdate and nitrate inhibited the enzyme, although less severely than perchlorate, sulfite, chlorate and

Table 1. Screening of Oxyanions to Identify Inhibitors of Oxalate Oxidase

		Remaining Activity (%) [1]			
Compound	**In Assay**	**0.1 mM**	**1 mM**	**10 mM**	**20 mM**
Arsenate	$H_2AsO_4^-$	100	100	95	67
Arsenite	$HAsO_2$	100	100	100	100
Carbonate	H_2CO_3	100	100	83	52
Chlorate	ClO_3^-	ND[2]	8	NDTD[3]	ND
Chlorite	ClO_2^-	85	53	17	7
DTPA	di-anion	100	98	86	78
EDTA	di-anion	100	100	99	93
Molybdate	$HMoO_4^-$	100	78	NDTD	NDTD
Nitrate	NO_3^-	44	17	9	7
Nitrite	NO_2^-	99	8	6	NDTD
Perchlorate	ClO_4^-	ND	NDTD	NDTD	NDTD
Phosphate	$H_2PO_4^-$	100	100	95	78
Selenate	SeO_4^{2-}	100	100	85	49
Sulfite	HSO_3^-	ND	NDTD	ND	ND

[1]The rate of degradation of oxalic acid by oxalate oxidase with no inhibitor present (i.e. the rate corresponding to 100%) was 0.012 ± 0.001 mM/min. [2] ND = not determined. [3]NDTD = none detected.

nitrite. The remaining activity at an inhibitor concentration of 1 mM was 53%, 78% and 17% for chlorite, molybdate and nitrate, respectively. Molybdate has been shown to inhibit oxalate oxidase from *Bougainvillea spectabilis* and 40% of the activity remained at a concentration of 1 mM [13]. The remaining activity at a molybdate concentration of 1 mM was slightly higher for oxalate oxidase from barley (Table **1**). Nitrate has been reported to inhibit oxalate oxidase, but the literature shows divergent results. Sugiura *et al*. [14] reported that 16% of the activity of oxalate oxidase from barley seedlings remained at a nitrate concentration of 1 mM. In contrast, Kotsira and Clonis [10] reported that 97% of the activity of oxalate oxidase from barley roots remained at a nitrate concentration of 1 mM. Our result (17% remaining activity, Table **1**) resembles that of Sugiura *et al*. [14].

Arsenate, arsenite, carbonate, EDTA, phosphate and selenate did not inhibit oxalate oxidase at a concentration of 1 mM. DTPA had only a minor inhibitory effect and 98% of the activity remained at 1 mM. At a concentration of 3.3 mM, arsenate did not inhibit oxalate oxidase from *Bougainvillea spectabilis* [13]. The results presented here (Table **1**) show that arsenate had only minor inhibitory effect on oxalate oxidase from barley seedlings at concentrations at or above 10 mM.

Kinetic Studies and Mechanism of Inhibition

The apparent K_M of barley oxalate oxidase was 0.28 ± 0.05 mM. This result is very similar to the result reported for oxalate oxidase from barley roots which had a K_M value of 0.27 mM at 25°C and pH 3.8 [10]. The Michaelis-Menten plots (not shown) indicate that there was no substrate inhibition in the substrate concentration range studied.

Chlorate, chlorite and perchlorate were chosen for studies of the mechanism of inhibition. Chlorate, which may occur in bleaching filtrates from processes based on bleaching of pulp with chlorine dioxide, is known to be a potent inhibitor of oxalate oxidase [12], but the mechanism through which the enzyme inhibitors act has not been studied previously. The K_i and K_i' values were calculated as described by Cornish-Bowden [16]. The results are shown in Fig. **1** and summarized in Table **2**. The K_i and K_i' values with chlorate as inhibitor were 0.031 ± 0.002 mM and 0.35 ± 0.01 mM, respectively. With chlorite, K_i was 0.7 ± 0.1 mM and K_i' was 7.0 ± 0.7 mM. With perchlorate, K_i was 0.0032 ± 0.0003 mM and K_i' was 0.010 ± 0.001 mM.

The results show that the inhibition caused by chlorate, chlorite and perchlorate becomes more severe as the oxidation state increases. Furthermore, the results indicate that all

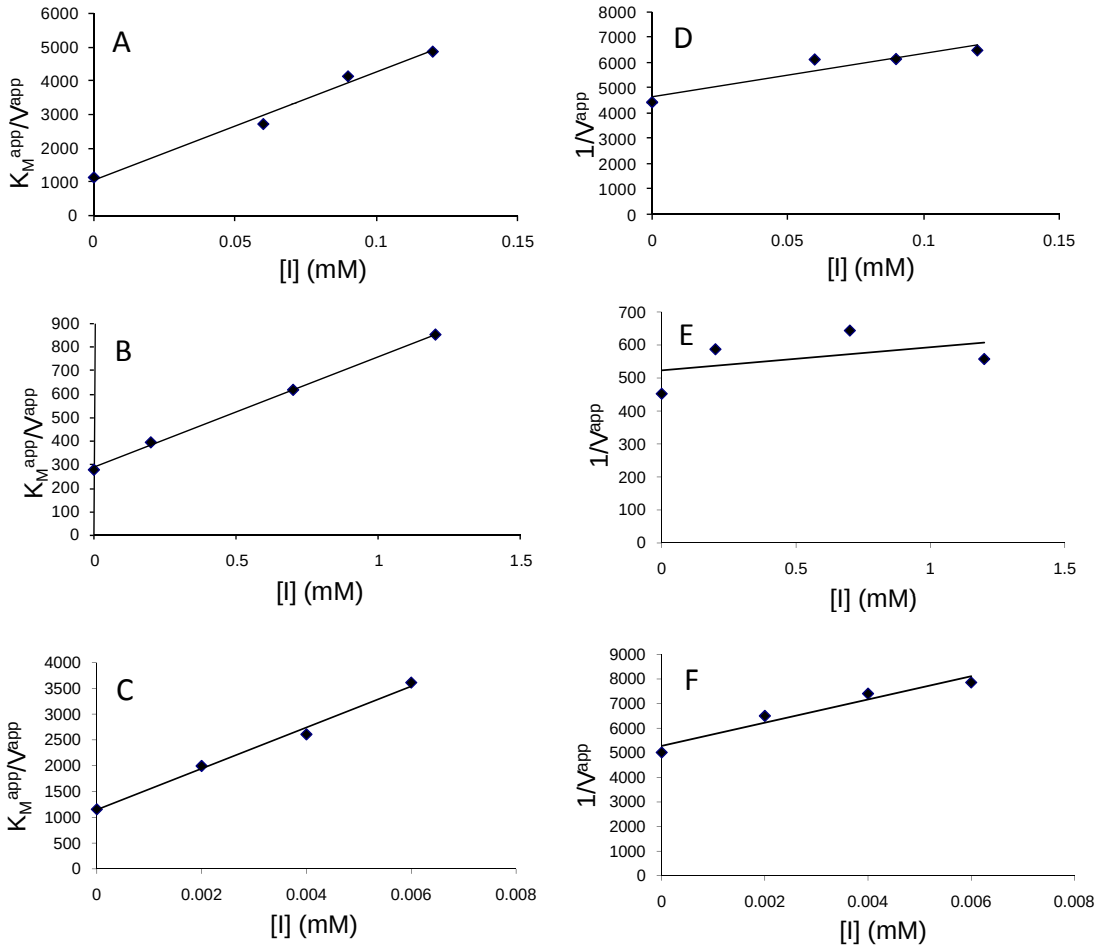

Fig. (1). Secondary plots for determination of K_i [(**A**) chlorate, (**B**) chlorite, (**C**) perchlorate] and K_i' [(**D**) chlorate, (**E**) chlorite, (**F**) perchlorate].

Table 2. Kinetic Studies of Inhibition of Oxalate Oxidase

Inhibitor	K_i (mM)	K_i' (mM)	Inhibition Type
Chlorate	0.031 ± 0.002	0.35 ± 0.01	Mixed
Chlorite	0.7 ± 0.1	7.0 ± 0.7	Mixed
Perchlorate	0.0032 ± 0.0003	0.010 ± 0.001	Mixed

three compounds act as mixed inhibitors of oxalate oxidase. For all three inhibitors, the K_i' values are higher than the K_i values (Table **2**). The results suggest that the inhibitors in part act by interfering with the binding of the substrate to the enzyme as indicated by K_i values that are sometimes about ten times lower than the corresponding K_i' values (Table **2**).

Properties and Effects of Oxyanions

The charges and the hydrodynamic radii of the oxyanions might be of importance for their ability to inhibit oxalate oxidase. The pKa values for oxalic acid are pKa_1=1.23 and pKa_2=4.19 [17], which indicates that at the pH of the assay, namely 3.8, oxalic acid predominantly occurs as the mono-anion. The hydrogen oxalate ion has a hydrodynamic radius of 2.29×10^{-10} m.

The oxyanions chlorate, chlorite, molybdate, nitrate, nitrite, perchlorate and sulfite were all inhibitors of oxalate oxidase from barley at a concentration of 1 mM. Their hydrodynamic radii vary between 1.23×10^{-10} m and 1.77×10^{-10} m [17]. Oxyanions that showed none or negligible inhibitory effect at 1 mM included arsenate, DTPA, EDTA, phosphate and selenate. They all have in common that their hydrodynamic radii are $\geq 2 \times 10^{-10}$ m. It is possible that the relatively large radii of these anions contribute to making them less powerful as inhibitors of oxalate oxidase by rendering interaction with the active site of the enzyme more difficult. Even though oxalic acid has a radius that is slightly larger than 2×10^{-10} m, the single bond between the two carboxyl groups makes the molecule flexible. Since oxalic acid is a mono-anion at pH 3.8, the hydrogen atom can appear at any of the carboxylic groups, each of which is a conjugated system. This also contributes to a high flexibility. The active site and its monodentate binding of glycolate has been investigated [6, 18], but the interaction between the enzyme and potent inhibitors positioned in the active site remains to be elucidated.

Selenate is a di-anion and that could possibly contribute to its inability to inhibit oxalate oxidase at 1 mM (Table **1**). At the pH of the assay, arsenite and carbonate are both protonated (i.e. they occur as arsenenous acid and carbonic acid) and that might explain why these compounds did not interfere with the activity at 1 mM.

Inhibitors in Industrial Applications

Among the oxyanions studied, carbonate, chlorate, chlorite, DTPA, EDTA and sulfite are of particular interest for application of oxalate oxidase in industrial processes, since these ions may occur in process water from the pulp and paper industry [12,19,20]. DTPA and EDTA have been correlated with low enzyme activity [20]. In that study, DTPA and EDTA were present in filtrates in concentrations up to 74 μM and 10.4 μM, respectively. According to the results presented here, these concentrations are not high enough to inhibit the enzyme, but as DTPA and EDTA act as chelators of metal ions, they may have other effects.

CONCLUSIONS

Seven out of fourteen tested oxyanions and related compounds had an inhibitory effect on oxalate oxidase at a concentration of 1 mM. The results suggest that the size and charge of the compounds are important for their inhibitory effect. Our results, the first mechanistic investigation of inhibitors of oxalate oxidase, show that chlorate, chlorite and perchlorate cause mixed inhibition of the enzyme and that the inhibitory effect of these oxyanions increases with increasing oxidation state. Studies of the action of inhibitors of oxalate oxidase are of relevance for understanding the properties of the enzyme and for enzymic applications in industry and medicine.

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