Barley (*Hordeum vulgare*) oxalate oxidase is a manganese-containing enzyme

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Oxalate oxidase (EC 1.2.3.4) catalyses the conversion of oxalate and dioxygen into CO₂ and H₂O₂. The barley (*Hordeum vulgare*) seedling root enzyme was purified to homogeneity and shown by metal analysis and EPR spectroscopy to contain Mn(II) at up to 0.80 atom per subunit. The involvement of Mn and neither flavin, Cu nor Fe in the direct conversion of dioxygen to H₂O₂

makes oxalate oxidase unique. A model of the active site of the holoenzyme based on a homology model of the apoenzyme is proposed.

Key words: EPR, germin, homology model, hydrogen peroxide.

INTRODUCTION

Oxalate oxidase (EC 1.2.3.4) catalyses the conversion of oxalate and dioxygen to CO₂ and H₂O₂ [1]. It is used for the clinical detection of oxalate in urine and plasma. The enzyme activity has been detected in several species, with the barley enzyme being the best characterized [2,3]. The barley enzyme has been shown to be 96% identical with a wheat (*Triticum*) protein called germin [4,5]. Although both barley oxalate oxidase and wheat germin belong to a large family of germin-like proteins that have also been termed cupins [6], only these two members of the family have been unambiguously shown to possess oxalate oxidase activity. Barley oxalate oxidase is an apoplasmic glycoprotein [7] that was thought to be a pentamer, but now appears more likely to be a hexamer of ≈ 25000 Da subunits [8]. The enzyme activity is detected in barley seedling roots during development and in the leaves of mature plants in response to powdery-mildew infection [9,10]. It has been suggested that the H₂O₂ that is produced by the enzyme is used in cell-wall cross-linking, as a fungicide or as a hormone. The enzyme's potential role in fighting fungal infection has led to much effort towards the generation of transgenic plants with higher oxidase activity and increased fungal resistance [6].

There are a limited number of cofactors that are known to be utilized by oxidases to effect the conversion of O₂ directly into H₂O₂. There are, for example, many well known examples of oxidases, such as glucose oxidase, that utilize flavins. Other oxidases utilize Cu(II), including amine oxidase-type enzymes [11] and galactose oxidase. Two oxidases that appear to contain neither flavin nor copper are a non-haem-iron-containing secondary alcohol oxidase [12] and a cobamide-containing ethanolamine oxidase [13]. In principle, haem-Fe(III) could also convert O₂ directly to H₂O₂.

Despite early suggestions that barley oxalate oxidase requires flavin for activity, purified and active enzyme preparations do not exhibit the UV/visible spectrum of flavoproteins [2,3]. An apparent increase in oxalate oxidase activity on the addition of flavin was probably due to the direct photochemical oxidation of oxalate by flavin [14]. With an absence of flavin, the enzyme would therefore be expected to contain a metal-ion cofactor. There are a number of germin-like proteins in the cupin super-

family which bind metal ions such as the Fe- or possibly Zn-containing phosphomannose isomerases and the Fe-containing dioxygenases [15]. A homology model of the barley enzyme indicates the clustering of three well-conserved histidine residues of the germin family that could bind a metal-ion cofactor in the centre of a β -barrel fold [16]. There are reports that suggest the metal-ion cofactor could be copper [1,17] or Fe [18,19], but none provide any direct evidence. The activation of the barley enzyme by Ca²⁺ and Pb²⁺ salts has also been reported [3]. However, Ca²⁺ could only have a structural role, and activation by Pb²⁺ could have been due to the known chemical oxidation of oxalate by Pb salts under acidic conditions [20]. There is one report of an apparently unrelated microbial oxalate oxidase that contains Mn [21].

We show in the present paper that the enzyme purified from barley seedling roots grown in this laboratory contains only mononuclear Mn(II) and none of the other candidate cofactors. This result is discussed in terms of its physiological significance, the catalytic mechanism and the possible structure of the active site using an alternative homology model.

EXPERIMENTAL

Materials

All chemicals and biochemicals were of the highest grade available and, unless otherwise stated, were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.). Sodium dithionite was purchased from BDH Chemicals (Poole, Dorset, U.K.). Recleaned barley seeds were obtained from the Plant Breeding Institute Cambridge Ltd. (Cambridge, U.K.). Horseradish peroxidase (HRP4C) was purchased from Biozyme Laboratories Ltd. (Blaenavon, Gwent, Wales, U.K.). Distilled deionized water was used throughout. Commercial barley seedling oxalate oxidase was purchased from Boehringer-Mannheim.

Methods

Acetate buffer refers to 50 mM sodium acetate buffer, pH 5.0, unless stated otherwise. A Perspective Biosystems (Hertford, U.K.) Voyager-DE STR spectrometer was used for matrix-assisted

Abbreviations used: ABTS, 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulphonic acid); BCA, bicinchoninic acid; MALDI-TOF, matrix-assisted laser-desorption-ionization time-of-flight.

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laser-desorption-ionization time-of-flight (MALDI-TOF) MS at the Department of Biochemistry, University of Dundee, Dundee, Scotland, U.K. Metal analysis was provided by Southern Science Laboratories (Lewes, East Sussex, U.K.) using inductively coupled plasma spectroscopy of acid-digested samples. Protein samples were dialysed before digestion and the resulting dialysis buffers were used as control samples. Metal content is quoted after subtraction of the control values. UV/visible spectra and time courses were obtained using Shimadzu UV-210 IPC and MPS-2000 spectrophotometers respectively, where the path-length was 1 cm. Protein was determined using the Pierce Micro bicinchoninic acid (BCA) protein assay and enzyme molarity was calculated using the predicted protein molecular mass of 21 203 Da.

Hydroponic barley seedling growth

Surface-sterilized seeds were thoroughly washed and soaked in cold water for between 2 and 4 h before hydroponic growth at 22 °C. After 2 days, the water was continuously and rapidly aerated. After between 6 and 10 days, the seedling roots were cut away from underneath the mesh screen and immediately frozen using liquid nitrogen and stored at -20 °C. A number of barley varieties were grown hydroponically, and crude extracts of 6-day-old roots were prepared. Using the criteria of oxalate oxidase units per seed, specific activity, germination efficiency, yield of roots and lack of root infection, the barley variety 'Dandy' was selected as the best overall.

Enzyme purification

Frozen roots were ground with a chilled pestle and mortar and then thawed in the presence of cold acetate buffer (3:1, v/w) containing polyvinylpyrrolidone (1%, w/v). After 1 h incubation at 4 °C, the homogenate was squeezed through a double layer of Miracloth (Calbiochem) and the filtrate was centrifuged (1 h, 13 000 g, 4 °C) giving a crude extract. Oxalate oxidase was purified from the extract using a method based on that developed by Kotsira and Clonis [3] with modifications. The extract was heat treated (60 °C for 10 min) and centrifuged to remove precipitated protein. The supernatant was concentrated by diafiltration and dialysed against acetate buffer containing NaCl (0.1 M), CaCl₂ (1 mM) and MgCl₂ (1 mM). The protein was applied to a concanavalin A-Sepharose 4B column that had previously been equilibrated with the same buffer. The enzyme was eluted with methyl α -D-mannopyranoside (2 mM) and was dialysed against acetate buffer (10 mM). The protein was applied to a carboxymethyl-Sepharose column that had been previously equilibrated with this buffer. The enzyme was eluted with 100 mM NaCl using a 0–500 mM gradient. The protein was applied to a Superdex 200 HR 10/30 column (Pharmacia) that had previously been equilibrated with acetate buffer containing NaCl (1.5 M). Typically, 650 g of barley seedling roots gave 23 g of protein and 53 units of oxalate oxidase in the crude extract which gave 13 units of purified enzyme. One unit of enzyme activity is defined as that capable of producing 1 μ mol of H₂O₂/min. The specific activity determined at 22 °C (10–13 units/mg of protein) compared well with that reported by others using similar, but not identical, assays at 37 °C (9 [4] and 34 units/mg [3]).

Enzyme assays

The formation of H₂O₂ by oxalate oxidase was coupled to the horseradish peroxidase-mediated oxidation of 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS). The assays were

monitored by an increase in absorbance at 650 nm at 22 °C (ϵ 10 000 M⁻¹·cm⁻¹ for the ABTS radical product under these conditions). Assay mixtures contained 50 mM sodium succinate buffer, pH 4.0, with horseradish peroxidase (24 units; as defined by supplier), ABTS (5 mM), potassium oxalate (20 mM) and oxalate oxidase, which was added last, in a total volume of 1 ml. The enzyme was equally active in citrate and succinate buffers. The pH optimum of the enzyme was determined to be 4.0 using citrate buffers with an oxygen electrode.

PAGE

The PhastSystem (Amersham Pharmacia Biotech) was used for all PAGE using the protocols described in the PhastSystem manual and method files. PhastGel gradient (8–25% polyacrylamide) gels were used for both SDS and native PAGE. Samples for SDS/polyacrylamide gels destined for oxalate oxidase activity staining were prepared without the addition of dithiothreitol and without heat treatment. PhastGel isoelectric-focusing gels, pH 3–9, were used.

EPR spectroscopy

X-band ($\approx$ 9.4 GHz) EPR spectra were recorded on a modified Bruker ER 200D SRC EPR spectrometer (Bruker Spectrospin), fitted with an ESR-900 liquid-helium flow cryostat (Oxford Instruments) and run with Bruker software on a Bruker ESP 1600 computer. Conditions were adjusted to ensure non-saturation of signals. Spectra were obtained with a microwave frequency and power of 9.427 GHz and 0.2 mW with a receiver gain of 3.2×10^5 at 30 K. Baseline corrections were made using identical quartz tubes containing only buffer under identical conditions. Q-band ($\approx$ 35 GHz) EPR spectra were recorded on a Varian E-9 EPR spectrometer at 77 K, and baseline corrections were made as stated above.

Homology modelling

The structure of barley oxalate oxidase was modelled on the basis of similarity to the C-terminal domain of jack-bean (*Canavalia ensiformis*) 7 S vicilin, canavalin (Icau [22]), in a manner similar to that used by Gane et al. [16]. The sequences of oxalate oxidase (accession number P45850) and the C- and N-terminal domains of both canavalin (P50477) and phaseolin (P02853, P07220) were aligned using the CLUSTAL W 1.7 algorithm [23]. The alignment in regions of poor identity and similarity was adjusted by comparing the predicted secondary structure of oxalate oxidase using the PHD algorithm [24] with the known secondary structures of canavilin and phaseolin (2phl [25]). The homology model was generated with the adjusted alignment using Swiss-PdbViewer 3.0 and energy-minimized using SWISS-MODEL [26]. Mn(II) was modelled into the proposed active site with molecular-mechanics MM2 and molecular-dynamics energy minimization using CS Chem3D Pro 3.5 (a computational force-field program). Figures were generated using RasMac 2.6.

RESULTS

Properties of the purified enzyme

The purified enzyme appeared as a single band with a molecular mass of 26 000 Da on silver-stained SDS/polyacrylamide gels.

Table 1 Metal analysis of oxalate oxidase purified from barley seedling roots

Metals were analysed using inductively coupled plasma spectroscopy, and protein was determined using the Pierce Micro BCA protein assay using a protein molecular mass of 21 203 Da as predicted from the primary sequence.

Specific activity (units/mg)	Metal ...	Metal atoms per oxalate oxidase monomer			
		Cu	Mn	Fe	Zn
10		0.01	0.52	0.14	0.60
13		0.03	0.80	0.00	0.24

This subunit mass is very similar to that determined for the commercial enzyme and that reported by others [3,9,10,27]. However, this analysis appears to overestimate the subunit size, because MALDI-TOF MS gave a value of 22098 ± 55 Da. Since the molecular mass of the protein predicted from the primary sequence is 21 203 Da, the glycan must have a molecular mass of about 895 Da, assuming no other post-translational modifications have occurred. The N-linked glycans associated with the G [GlcNAc(Fuc)GlcNAcMan(Xyl)(ManGlcNAc)₂] and G' [GlcNAc(Fuc)GlcNAcMan(Xyl)(Man)₂] forms of wheat germin [7] are predicted to have masses of 1578 and 1171 Da respectively. Therefore, the glycan associated with the barley enzyme would appear to be most similar to that of the smaller G' form and could be smaller still. The oligomeric protein tended to precipitate during native PAGE. However, if the sample is not boiled before SDS/PAGE, it has an apparent molecular mass of 80 000 Da, and its activity can be detected *in situ*, as reported by others [10]. Gel filtration gave a value for the active oligomer of 108 000 Da, which is closer to that expected for a hexamer (132 600 Da), but is still an underestimate. The pI of the protein was determined to be 6.9, which is higher than that of 5.7 predicted from the primary sequence. A K_m of 1.3 mM and a k_{cat} of about 10 s^{-1} were determined at pH 4.0 and 22 °C, with no indication of substrate inhibition at oxalate concentrations of up to 200 mM. Other groups report the slightly lower K_m values of 0.42 [28] and 0.27 mM [3] and the observation of substrate inhibition at concentrations of ≥ 4 mM.

UV/visible spectroscopy

Purified and active oxalate oxidase is colourless. It has a UV/visible spectrum with an essentially normal protein peak ($\lambda_{max} = 278 \text{ nm}$). From the primary sequence it is predicted that the absorbance at 278 nm would be 1.218 at a concentration of $145 \mu\text{M}$ ($\epsilon = 8400 \text{ M}^{-1} \cdot \text{cm}^{-1}$ [29], assuming all cysteine residues are free [3]). Since the observed absorbance of 1.252 is 1.03-fold, and thereby very close to the expected value, there is no indication of an additional chromophore. The spectrum confirms the lack of oxidized flavin or a related pterin in the purified and active enzyme. Furthermore, enzyme that has been denatured with urea and heat treatment does not liberate any reduced flavin or pterin forms that would become oxidized on contact with air. There is no indication of ferric haem, cobamide, a galactose oxidase-like Cu(II) ion, a secondary alcohol oxidase-like non-haem Fe or superoxide dismutase-like Mn(III). Although the presence of flavin, haem and cobamide can be ruled out, the presence of either copper, Mn(II) or non-haem Fe cannot.

Figure 1 X-band EPR spectrum of oxalate oxidase

The Figure shows the X-band EPR spectrum of oxalate oxidase ($145 \mu\text{M}$) in acetate buffer at 30 K, indicating a six-line protein-bound Mn(II) spectrum at $g \approx 2.0$ and a small but significant signal at $g \approx 4.4$. The inset shows the six-line spectrum of MnCl_2 (0.02 mM) that has no additional signals in acetate buffer under identical conditions.

Figure 2 Q-band EPR spectrum of oxalate oxidase

The Figure shows the Q-band EPR spectrum of oxalate oxidase ($998 \mu\text{M}$) at 77 K indicating the typical six-line protein-bound Mn(II) spectrum at $g \approx 2.0$ and a less typical feature at $g \approx 2.5$. The inset shows a spectrum of the same sample with a narrower sweep width.

Metal analysis

Enzyme purification involves three chromatographic and two dialysis steps, allowing the removal of extraneous metal ions. None of the metal ions that the protein was exposed to during the purification (Na^+ , Ca^{2+} and Mg^{2+}) were candidate cofactors. The metal content of two preparations of the purified enzyme was determined (Table 1). None of the samples contained

Figure 3 Homology model of proposed active site of oxalate oxidase

(A) A homology model of barley oxalate oxidase based on the known canavalin crystal structure showing the clustering of a conserved glutamate residue with the three conserved histidine residues. The local protein fold is indicated with an α -carbon trace. (B) A molecular model showing the potential for the glutamate residue, histidine residues and two water molecules to ligate a Mn(II) ion with octahedral geometry without any change in the positions of the α - and β -carbon atoms. The angle of view is essentially the same as that in (A). The water molecules are oriented such that they occupy the cavity of the β -barrel fold of the model and have direct access to solvent through an opening at one end of the model which is perpendicular to the plane of the Figure.

significant amounts of Cu. It is clear that the Mn content increased with increasing specific activity of the enzyme, up to a maximum of 0.80 atom/subunit. Conversely, the Fe content decreased with increasing specific activity and was not detected in the most highly purified preparation. The Zn content decreased in a similar manner. It is noteworthy that the combined Mn and Zn content was close to 1 atom per monomer with both batches of purified enzyme. It is likely that the small differences in specific activity between batches are not due to contaminating protein, since each appeared to be pure according to SDS/PAGE with silver staining. The differences may therefore be due to variable substitution of Mn(II) ions by Zn(II) ions. The presence of other metal ions, such as Ca^{2+} , which could have a structural role, cannot be ruled out. The preincubation of the purified enzyme with MnCl_2 led to no increase in enzyme activity. This indicates either that the enzyme has its full complement of Mn(II) or that it does not reversibly bind the ion in the fully folded form. The latter would seem to be likely, since exhaustive dialysis in the absence of Mn(II) salts leads to no loss of activity. The commercial enzyme, when further purified, gave similar results overall, with no Pb ions being detected.

EPR spectroscopy

The X-band EPR spectrum of oxalate oxidase exhibits a typical six-line mononuclear Mn(II) spectrum at $g \approx 2.0$ (Figure 1). The Mn is clearly protein-bound, since it does not resemble the spectrum of MnCl_2 in the same buffer (inset to Figure 1). The intensity of the spectrum is consistent with the concentration of protein in the sample. However, the poor recovery of the baseline at high field, due to some broad underlying feature, makes it difficult to integrate the spectrum precisely. There is an additional signal at $g \approx 4.4$ that is not present in MnCl_2 spectra. This can be interpreted as being associated with a Mn(II) ion with unusually moderate zero-field splitting parameters. The Q-band EPR spectrum shows the six-line Mn(II) spectrum at $g \approx 2.0$ without the underlying broad feature, but with the low-field signal now at $g \approx 2.5$ (Figure 2). The addition of sodium dithionite (2 mM) to the sample led to less than a 5% increase in the amplitude of the X-band signals, indicating that the resting state of the enzyme is the Mn(II) form. Neither the reduced nor the as isolated states show the presence of Fe(III), Cu(II), Ni(III) or Co(II) in the X- and Q-band EPR spectra.

Molecular modelling

A homology model was generated using essentially the same procedure as Gane et al. [16], except that the barley oxalate oxidase was modelled on to only the C-terminal domain of canavalin rather than the wheat enzyme on a protein scaffold generated from both the N- and the C-terminal domains of the seed storage proteins canavalin and phaseolin. The C-terminal domain of canavalin was chosen because of its higher sequence identity and similarity to oxalate oxidase in the conserved germin motifs. The homology model shows the clustering of the three highly conserved histidine residues (Figure 3A) in a β -barrel fold, as seen by Gane et al. [16]. However, this model also shows the clustering of an almost equally highly conserved glutamic acid-95 (E⁹⁵) with the histidine residues. Although Gane et al. [16] note that E⁹⁵ lies close to the histidine cluster in their model, they do not include it in their cluster. Chemical-modification studies have indicated that a carboxylate side chain is essential for activity, and this may be E⁹⁵ [30]. Our model is attractive because all of the residues that are at least partially conserved in the germin motifs [15] and are potential metal ligands are now clustered together. In addition, there are no other candidate ligands in the vicinity of the cluster within the cavity of the β -barrel fold. Furthermore, there is precedence in superoxide dismutase for a Mn ion to be ligated by three histidine residues and a carboxylate side chain [11].

There are no reports that canavalin and phaseolin can bind metal ions. However, they possess neither the glutamate nor all of the histidine residues of the germin motifs. The potential for these residues of the oxalate oxidase model to ligate a Mn(II) ion was explored by molecular modelling. The proposed ligands histidine-88 (H⁸⁸), H⁹⁰, E⁹⁵ and H¹³⁷ were ligated to an octahedral Mn(II) ion through their ϵ -nitrogen atoms and the carboxy oxygen. The remaining two primary co-ordination sites were filled with two water molecules that faced the cavity of the β -barrel, one of which would be expected to be deprotonated to give net charge neutralization. These components of the model were then energy-minimized while keeping the α - and β -carbon atoms of the four amino acids static. This resulted in a model of the active site of the holoenzyme (Figure 3B). The bond lengths and bond angles were essentially normal. There were no clashes with any other components of the model, since the four key residues are situated within a cavity large enough to accept the metal ion with two water molecules and all have adjacent residues that are either proline residues or whose side chains are situated on the exterior of the β -barrel fold. The water molecules ligated to the Mn ion have access to solvent through an opening at one end of the β -barrel fold, indicating the possible route that the substrates and products would take. The binding of the substrates to form a ternary complex would presumably result in the displacement of the two water molecules. There is an important difference between the geometry of the proposed model and that of superoxide dismutase. The carboxylate ligand is *trans* to the site of superoxide binding in superoxide dismutase, whereas the only possible geometry in the oxalate oxidase model involves the carboxylate ligand being *trans* to the H⁸⁸ amino acid side chain.

DISCUSSION

We have purified barley seedling root oxalate oxidase to homogeneity, confirmed its integrity and identified the cofactor to be a mononuclear Mn(II) ion with up to 0.80 atom/subunit. Since some of the germin-like proteins in the cupin superfamily are thought to bind other metal ions such as Fe and possibly Zn [6,15], it is possible that all members of the superfamily are

capable of binding specific metal ions that have distinct catalytic and structural roles. For example, the iron-containing 3-hydroxy-anthranilate-3,4-dioxygenases have an aspartate residue in place of one of the highly conserved histidine residues. Interestingly, there is a reference to a Mn-deficiency protein from tomato (*Lycopersicon esculentum*) that is a germin-like protein in the sequence data bases which may also turn out to bind Mn ions (DDBJ/EMBL/GenBank[®] accession number 2979494).

There is one report that an oxalate oxidase from a *Pseudomonas* species was found by metal analysis to contain Mn at an atom/subunit ratio of 1.12:1, with little or no detectable Cu, Fe, Zn, Mo, Co or Ni [21]. Although the plant and microbial enzymes therefore both contain Mn, they have distinctly different properties. For example, a 1.75-fold increase in enzyme activity on the addition of a Mn(II) salt to the microbial enzyme suggests that a total of 1.96 atoms of Mn/subunit are optimal. The microbial enzyme appears to be an octamer of 38 000 Da subunits. The pH optimum is 4.8, compared with ~4.0 for the barley enzyme. Finally, the pI is 4.7, whereas that for the barley enzyme is 6.9. A more detailed comparison cannot be made, since no EPR spectroscopy was reported, no sequence information is known, the organism was not deposited and a microbial enzyme has not been reported since. What is clear is that oxalate oxidases constitute a new class of enzyme that utilize Mn in the conversion of O₂ into H₂O₂.

The severity of take-all infection of wheat (caused by the fungus *Gaeumannomyces graminis*) is related to the Mn content of seed [31]. Since oxalate oxidase is expressed in cereals in response to fungal infection, and we have shown that the enzyme contains Mn, it is possible that the Mn required by wheat to resist take-all may be associated with oxalate oxidase activity. It may therefore not be a coincidence that Mn-efficient lines, which are more resistant to take-all, tend to be more efficient at converting phenolics into lignin [32], since the H₂O₂ produced by oxalate oxidase could be used in lignin cross-linking. It is likely that, if Mn were limiting in seed and soil, transgenic crops with elevated oxalate oxidase expression levels may not exhibit higher enzyme activities and may not fulfil their potential for increased fungal resistance.

Given the absence of not only flavin and Cu, but also Fe and cobamide, in barley oxalate oxidase, it would seem highly likely that the Mn(II) ion is responsible for catalysis, rather than just having some structural role. This is feasible, since the well-studied permanganate-oxalate reaction can be coupled to H₂O₂ formation [33]. Rather than oxalate being degraded in the initial reactions involving the reduction of permanganate to Mn(III), it reacts only with the Mn(III), leading to the formation of Mn(II), CO₂ and a formyl radical. The formyl radical is then capable of reacting with O₂ and the Mn(II) ion under acid conditions to form H₂O₂ and CO₂, while regenerating the Mn(III) ion. The sum of these reactions is the same as the oxalate oxidase reaction. Such solution chemistry has been suggested to be involved in wood rotting by fungi [34]. Despite the attraction of this type of chemistry, it would seem to be unlikely that the enzyme utilizes this in catalysis. This is because the enzyme, as isolated, contains Mn(II), with essentially no Mn(III). One reason why the enzyme may not use the above chemistry is that it could be detrimental to generate formyl radicals in the absence of O₂.

An alternative catalytic cycle that involves the enzyme in the Mn(II) resting state is possible. The first step would be the binding of oxalate to the Mn(II) ion. This could lead to the lowering of the reduction potential of the Mn(III)-Mn(II) couple to below -0.16 V to allow the binding of O₂ as superoxide, with concomitant oxidation to Mn(III). This cycle therefore has the advantage that the enzyme will not undergo any radical chemistry

until both substrates are present. The successive decarboxylation of oxalate would generate a Mn(II) peroxy species that would produce H_2O_2 after protonation. The unusual EPR spectrum of the enzyme and the detection of transient Mn(III) and radical species will be the subject of further study which will help to elucidate the role of the metal ion in catalysis.

The homology model of oxalate oxidase based on the C-terminal domain of canavalin provides an attractive model of an active site that is capable of binding the Mn(II) ion. The proposed active site superficially resembles that of superoxide dismutases that require Mn (and others that require Fe) [11]. There are a number of things that oxalate oxidase and Mn superoxide dismutase have in common. For example, both enzymes produce H_2O_2 . In addition, the Q143N (Glu¹⁴³ → Asn) mutant of the human dismutase which stabilizes the Mn(II) form, like oxalate oxidase, has no features in the UV/visible spectrum [35]. Finally, the dithionite-reduced form of *Escherichia coli* superoxide dismutase [36] and the Q143N human enzyme, exhibit protein-bound Mn(II) EPR signals. However, the EPR spectra are quite different to that of oxalate oxidase. A further difference is that the dismutase stabilizes the Mn(III) oxidation state in the resting state of the enzyme, with a reduction potential of about +0.31 V, unlike oxalate oxidase, which clearly stabilizes the Mn(II) form. One important difference between the model and the dismutases is the geometry of the amino acid side-chain ligands about the metal ion. This may well be an important factor in determining the reduction potential of the Mn ion and its specific catalytic properties. We eagerly await a crystal structure which will help confirm this hypothesis.

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