Oxalate Decarboxylase Requires Manganese and Dioxygen for Activity

OVEREXPRESSION AND CHARACTERIZATION OF BACILLUS SUBTILIS YvrK AND YoaN

Received for publication, July 30, 2001, and in revised form, August 21, 2001
Published, JBC Papers in Press, August 23, 2001, DOI 10.1074/jbc.M107202200

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The Bacillus subtilis oxalate decarboxylase (EC 4.1.1.2), YvrK, converts oxalate to formate and CO2. YvrK and the related hypothetical proteins YoaN and YxaG from B. subtilis have been successfully overexpressed in Escherichia coli. Recombinant YvrK and YoaN were found to be soluble enzymes with oxalate decarboxylase activity only when expressed in the presence of manganese salts. No enzyme activity has yet been detected for YxaG, which was expressed as a soluble protein without the requirement for manganese salts. YvrK and YoaN were found to catalyze minor side reactions: oxalate oxidation to produce H2O2; and oxalate-dependent, H2O2-independent dye oxidations. The oxalate decarboxylase activity of purified YvrK was O2-dependent. YvrK was found to contain between 0.86 and 1.14 atoms of manganese/subunit. EPR spectroscopy showed that the metal ion was predominantly but not exclusively in the Mn(II) oxidation state. The hyperfine coupling constant (A = 9.5 millitesla) of the main g = 2 signal was consistent with oxygen and nitrogen ligands with hexacoordinate geometry. The structure of YvrK was modeled on the basis of homology with oxalate oxidase, canavalin, and phaseolin, and its hexameric oligomerization was predicted by analogy with proglycinin and homogentisate 1,2-dioxygenase. Although YvrK possesses two potential active sites, only one could be fully occupied by manganese. The possibility that the C-terminal domain active site has no manganese bound and is buried in an intersubunit interface within the hexameric enzyme is discussed. A mechanism for oxalate decarboxylation is proposed, in which both Mn(II) and O2 are cofactors that act together as a two-electron sink during catalysis.

Oxalate decarboxylase (EC 4.1.1.2) converts oxalate to formate and CO2 (1). The enzyme has been detected in a number of fungi (1), including Agaricus bisporus (2), as well as in guinea pig liver (3). The secretion of oxalate by fungi appears to have several functions including pathogenesis, competition, controlling the availability of environmental nutrients, the detoxification of copper compounds, and lignocellulose degradation (1). The fungal oxalate decarboxylases are induced by oxalate and are thought to control excess oxalate concentrations. Oxalate decarboxylases have been used in the clinical assay of oxalate and could be used to decrease toxic oxalate levels in foods and in the environment (4). Recently, the enzyme has been shown to confer fungal disease resistance when expressed in plants (5), presumably through the degradation of the oxalate produced by pathogenic fungi to suppress the plant oxidative burst (6). We recently identified the first bacterial example of an oxalate decarboxylase from Bacillus subtilis (7). This bacterial enzyme, previously known as the hypothetical protein YvrK, is induced by acid pH and not by oxalate. Although its physiological function is not yet clear, it may be involved in the elevation of cytoplasmic pH, because the reaction involves the net consumption of a proton.

Oxalate decarboxylases belong to a large superfamily of proteins called cupins (8, 9). They share conserved motifs, most recently defined as GX5HXHX3,4EX6G and GX5PXGX2HX3N (10), and are predicted to share a conserved β-barrel fold (cupa is the Latin for small barrel). The three known sequences of verified oxalate decarboxylases are from Flammulina velutipes (formerly Collybia velutipes), Aspergillus phoenices, and B. subtilis (Fig. 1). The only other known closely related homologues are from Streptococcus mutans and Synechocystis sp., together with another hypothetical protein in B. subtilis, YoaN (4). The oxalate decarboxylases belong to the bcupic subunit of the cupin superfamily. The conserved motifs appear twice in their primary sequence, presumably arising from a gene duplication event during their evolution (9). The crystal structures of the bcupic seed storage proteins, canavalin, phaseolin, and proglycinin, clearly show domain duplication within each subunit (11–13). An additional hypothetical bcupic protein in B. subtilis, YxaG, does not cluster with the oxalate decarboxylases in phylogenetic analyses (4).

Either Schiff bases, formed between substrates and either lysine residues or pyridoxal phosphate, or thiamine diphosphate is normally utilized as the electron sink in the decarboxylation of 2-oxo acids. For example, oxalyl-CoA decarboxylase requires thiamine diphosphate for activity (14). Although the thioester of oxalate is susceptible to nucleophilic attack by the thiazolium ylide of this cofactor, the decarboxylate form of oxalate is not. Oxalate decarboxylases would therefore be expected to use a different cofactor(s). Unlike many other decarboxylases, they do not possess broad substrate specificity and are extremely specific for oxalate (1). Most importantly and intriguingly, the fungal enzymes require O2 for activity despite the reaction involving no net redox change (1, 7). No details about the cofactor requirement of oxalate decarboxylases have previously been reported.

The O2 dependence of oxalate decarboxylases indicates the

* This work was supported by a Competitive Strategic Grant from the Biotechnology and Biological Sciences Research Council to the John Innes Centre. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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This paper is available on line at http://www.jbc.org

Vol. 276, No. 47, Issue of November 23, pp. 43627–43634, 2001

Printed in U.S.A.
The aim of this work was to overexpress _B. subtilis_ YvrK, YoAN, and YxaG, to determine whether YoAN and YxaG were also decarboxylases, to identify the cofactor(s) required for activity, and to establish whether one or both of the potential active sites of each subunit are functional.

**EXPERIMENTAL PROCEDURES**

**Materials**—All chemicals and biochemicals were of the highest grade available and, unless stated otherwise, were purchased from Sigma-Aldrich. Sodium dithionite was purchased from BDH Laboratory Supplies (Poole, Dorset, UK). Metal analysis was provided by Southern Science Laboratories (Lewes, Sussex, UK), using inductively coupled plasma mass spectrometry of acid-digested samples. The metal content is quoted after the subtraction of control values obtained with buffer alone. UV-visible spectra and time courses were obtained using Hewlett-Packard HP8452A and Shimadzu MPS-2000 spectrophotometers, respectively, with path lengths of 1 cm. Protein was determined using the Bio-Rad dye-binding protein assay unless indicated otherwise. Purified YvrK concentration was determined using _ε_280 = 58,800 M⁻¹ cm⁻¹ (26). Values obtained using this method (relative value = 1.0) were only slightly lower than those obtained with the Bio-Rad protein assay (1.2) and the Pierce Micro bicinchoninic acid protein assay (1.0) using a protein molecular weight of 43,566 predicted from the primary sequence. The PhastSystem (Amersham Pharmacia Biotech) with 8–25% PhastGels was used for nondenaturing and SDS-polyacrylamide gel electrophoresis with Comassie staining. Jack bean urease (545 and 272 kDa) and bovine serum albumin (152 and 66 kDa) were used to calibrate nondenaturing gels.

**Bacterial Strains and Growth Conditions**—_B. subtilis_ 168, _Escherichia coli_ DH15a, and _E. coli_ BL21(DE3)pLysS cells were grown on Luria-Bertani medium (27). Carbenicillin (125 μg ml⁻¹), kanamycin (50 μg ml⁻¹), or chloramphenicol (100 μg ml⁻¹) was added as required.

**Cloning**—The open reading frames and associated ribosome binding sites of _B. subtilis_ 168 yvrK (GenBank™ accession no. 2653537), YoAN (GenBank™ accession no. 2619026), and YxaG (GenBank™ accession no. 563940) were aligned with those of _barley_ (Hordeum vulgare) oxalate oxidase (OxOx; GenBank™ accession no. 539054), _moss_ (Barbula unguiculata), tobacco nectarin 1 (GLSL1 and GLS2, GenBank™ accession no. 642923 and GenBank™ accession no. 6090829, respectively), and HvGfL1, a germin-like protein with nucleotide-sugar pyrophosphatase/phosphodiesterase activity (NSPP; GenBank™ accession no. 2612992). The intramotif spacing is indicated. Conserved residues are indicated with asterisks. The conserved residues, shown in bold, are known to ligate a Mn²⁺ ion in the active site of oxalate oxidase.

Involvement of a flavin, pterin, or metal ion in catalysis. The lack of any obvious chromophore suggests that neither flavin nor pterin is protein-bound (7). Many members of the cupin superfamily are known to require divalent metal ions for activity. One of the best characterized is oxalate oxidase from cereal crops (also known as germin), which converts oxalic acid and O₂ to CO₂ and H₂O. It is now known to bind mononuclear manganese predominantly in the Mn(II) oxidation state, utilizing the Glu and three His residues of the conserved cupin motifs together with two waters in a distorted octahedral coordination environment (Fig. 2) (15, 16). After manganese was identified in oxalate oxidase, two germin-like cupins with manganese-dependent superoxide dismutase activity were reported (17, 18). These enzymes share no sequence homology with the well known mitochondrial Mn-superoxide dismutases that are isolated in the Mn(III) oxidation state. Although one group has reported that germin possesses superoxide dismutase as well as oxalate oxidase activity (16), this finding remains to be confirmed by others. Other germin-like proteins have recently been identified as having nucleotide-sugar pyrophosphatase/phosphodiesterase activities (19). Although these enzymes could conceivably require a metal ion cofactor for activity, no analysis of this has yet been published.

The other cupins that are known to require metal ions for activity include the Fe(II)-dependent cysteine (20), 3-hydroxyanthranilate 3,4-dioxygenase (21), and homogentisate 1,2-dioxygenases (24), and the anthranilate 3,4- (21), 1-hydroxy-2-naphthoate (22), gentisate activity include the Fe(II)-dependent cysteine (20), 3-hydroxycofactor for activity, no analysis of this has yet been published. Although these enzymes could conceivably require a metal ion like proteins have recently been identified as having nucleo-
Enzyme Purification—Recombinant YvrK was purified using a method based on that used for the wild-type protein (7) but with modifications. All buffers used during the purification contained 10% glycerol and 10 mM MnCl₂, unless indicated otherwise. Thawed cells were broken with two passes through a French pressure cell in the presence of 50 mM Tris·HCl, pH 8.0, and DNase I. The cell debris was removed by centrifugation (25,000 × g, 30 min, 4 °C). The crude extract was applied to a DEAE-Sepharose FastFlow column (80-ml bed volume; Amersham Pharmacia Biotech) that was previously equilibrated with 50 mM Tris·HCl, pH 8.0. The decarboxylase assay was eluted with a 0–1 M NaCl gradient in pH 8.0 Tris buffer at 400 mM salt. The enzyme was dialyzed with 50 mM Tris·HCl, pH 7.0, and applied to an FPLC Mono Q HR10/10 column that was previously equilibrated with pH 7.0 Tris buffer. It was eluted with a 0–1 M NaCl gradient in pH 7.0 Tris buffer at 250 mM salt. The enzyme was precipitated with 95% ammonium sulfate and redissolved in the minimum volume of pH 7.0 Tris buffer containing 100 mM NaCl and 10 mM dithiothreitol but no MnCl₂. It was applied to a Sephadex 200 HR26/60 column and eluted with the same buffer without dithiothreitol. The column was calibrated using Sigma Gel Filtration Molecular Weight Markers with thyroglobulin used to determine the void volume. The purified enzyme was frozen in liquid nitrogen and stored at −80 °C. Thawed samples could be concentrated using ammonium sulfate precipitation as described above.

Enzyme Assays—One unit of enzyme activity is defined as the amount of enzyme required to convert 1 μmol of substrate to product in 1 min. Oxalate decarboxylase activity was assayed using the method of Magro et al. (30) at 26 °C. A mixture containing the enzyme and oxalate was incubated at pH 5.0 for 2 min before being neutralized with phosphate buffer, pH 9.5, to increase the pH to 7.5 to stop the decarboxylase reaction (7). Formate was determined spectrophotometrically at 7.5 using formate dehydrogenase with NAD. Formate production by oxalate decarboxylase was linear with time over a period of at least 20 min. The pH buffering capacity of oxalate when used at high concentrations (20–150 mM) required the use of up to 4 times the quantity of phosphate buffer to successfully neutralize the assay mixtures. Formate concentration was quantified by using the rearranged Michaelis-Menten equation after the determination of Kₘ and Vₘax for each batch of formate dehydrogenase in the conditions used in the assay. The values of Kₘ and Vₘax were determined by fitting the data with the Michaelis-Menten equation. Where indicated, solutions were made anaerobic using eight vacuum-N₂ gas flush cycles and transferred to a Belle Technology glove box (Portesham, Dorset, UK) maintained at <1 ppm of O₂. Residual dissolved O₂ was removed with the addition of either 1 mM dithionite or a combination of 4–8 mM glucose, 12 units of glucose oxidase, and 1,150 units of catalase at least 30 min before assay. Typically, 0.5 units of oxalate decarboxylase were added to the anaerobic assays. Oxalate oxidase activity was determined using a modified decarboxylase assay procedure. Instead of measuring formate with microbial assays, oxalate oxidase activity was determined using a modified procedure. Typically, 0.5 units of oxalate decarboxylase were added to the anaerobic assays. Oxalate oxidase activity was determined using a modified decarboxylase assay procedure. Instead of measuring formate with microbial assays, oxalate oxidase activity was determined using a modified procedure. Typically, 0.5 units of oxalate decarboxylase were added to the anaerobic assays. Oxalate oxidase activity was determined using a modified decarboxylase assay procedure. Instead of measuring formate with microbial assays, oxalate oxidase activity was determined using a modified procedure. Typically, 0.5 units of oxalate decarboxylase were added to the anaerobic assays. Oxalate oxidase activity was determined using a modified decarboxylase assay procedure. Instead of measuring formate with microbial assays, oxalate oxidase activity was determined using a modified procedure. Typically, 0.5 units of oxalate decarboxylase were added to the anaerobic assays. Oxalate oxidase activity was determined using a modified decarboxylase assay procedure. Instead of measuring formate with microbial assays, oxalate oxidase activity was determined using a modified procedure. Typically, 0.5 units of oxalate decarboxylase were added to the anaerobic assays. Oxalate oxidase activity was determined using a modified decarboxylase assay procedure. Instead of measuring formate with microbial assays, oxalate oxidase activity was determined using a modified procedure. Typically, 0.5 units of oxalate decarboxylase were added to the anaerobic assays. Oxalate oxidase activity was determined using a modified decarboxylase assay procedure. Instead of measuring formate with microbial assays, oxalate oxidase activity was determined using a modified procedure. Typically, 0.5 units of oxalate decarboxylase were added to the anaerobic assays.

Molecular Modeling—The structure of each domain of oxalate decarboxylase was modeled on the basis of homology with oxalate oxidase (Protein Data Bank (PDB) entry code 1fzi (16)) using the method described previously (15). The fold of the entire subunit and its trimerization were modeled on the basis of homology with the 7-s seed storage protein canavalin (PDB code 1cau (11)) and phaseolin (PDB code 2phl (12)). The dimerization of the trimer to give the hexamer was predicted by comparison with hexameric homogenate 1,2-dioxygenase (PDB code 1ey2 (24)) and the way in which proclycinin is thought to form hexamers (13).

Protein Expression—Because only 100-μg quantities of the oxalate decarboxylase YvrK can be purified from B. subtilis itself (7), it was cloned and overexpressed in E. coli. Although the recombinant protein was expressed at high levels under a variety of growth conditions, both the heat treatment of cells (42 °C, 2 min), just prior to the induction of expression and the addition of a Mn²⁺ salt to the growth medium on induction were necessary for the production of soluble and active enzyme. The addition of other metal ion salts (Fe²⁺, Cu²⁺, Ni²⁺, Co²⁺, Mg²⁺, Zn²⁺) did not give significant levels of active enzyme. This is the first example of an oxalate decarboxylase being successfully overexpressed in a microbial host. The specific requirement of Mn²⁺ for the correct folding and activity of YvrK strongly suggested that it may be a manganese-containing enzyme. Similarly, the overexpression of recombinant YoaG gave high levels of soluble protein only when the cells were heat shocked and a Mn²⁺ salt was added to the growth medium as described above. The addition of other metal ions gave less soluble protein (Mn²⁺ > Fe²⁺, Mg²⁺ > Co²⁺, Ni²⁺, Cu²⁺ > Zn²⁺). By contrast, recombinant YxaG was expressed in a soluble form without the need for heat shock or the addition of a metal ion.

YvrK Purification—Recombinant YvrK was purified using three column chromatography steps providing typically 15.8 mg of protein from 20 g of wet cell paste (Table I). The protein was at least 95% pure according to gel electrophoresis and had the expected subunit molecular mass of 44 kDa. Overall, the enzyme was purified 21-fold to give a specific activity (65 units mg⁻¹) that was consistently higher than that of the wild-type enzyme (26 units mg⁻¹ (7)), indicating the quality of the recombinant preparation. Gel filtration and nondenaturing polyacrylamide gel electrophoresis showed that the enzyme had a molecular mass of 254 and 222 kDa, respectively. This finding was similar to that reported for the wild-type enzyme (7) and strongly suggests that the active oligomer was composed of six subunits. The recombinant enzyme was occasionally prone to oxidation during the latter stages of the purification resulting in its dimerization, presumably because of the formation of interoligomer disulfides between cysteine residues, of which

| Step          | Volume | Protein | Activity | Specific activity | Fold purification | Yield |
|--------------|--------|---------|----------|-------------------|-------------------|------|
| Crude extract| 32     | 1171    | units    | 3700              | 1                 | 100  |
| DEAE-Sepharose| 48    | 362     | unit     | 32                | 1.3               | 9.3  |
| Mono Q       | 20     | 109     | unit     | 2230              | 20                | 6.5  |
| Ammonium sulfate | 6    | 57      | unit     | 1320              | 23                | 7.3  |
| Sephadex 200 | 15     | 15.8    | unit     | 1030              | 65                | 20.7 |

1 The abbreviations used are: ABTS, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid); mT, millitesla; PDB, Protein Data Bank.
clearly show the oxalate decarboxylase activity of YvrK to be activated by the presence of catalase. Overall, these results indicated that the enzyme was not containing catalase, but no glucose or glucose oxidase, gave O2 before assay regained 131% of its original activity. Assays of decarboxylation on O2. Enzyme that was treated with this dithionite (or an effect of its oxidation product sulfite) on the oxalate decarboxylase activity. Although this result was consistent with the O2 dependence of the reaction, dithionite is a powerful single-electron reductant that could have affected the enzyme in other ways. Enzyme that had been treated with dithionite and exposed to atmospheric O2 before assay regained enzyme activity. Emiliani and Riera (35) noted that when assaying the enzyme in the presence of this compound, the solution became pale yellow in color. We have observed a small but significant protective effect of this compound on YvrK decarboxylase activity during turnover, as well as the formation of a pale yellow color. The spectrum of the new compound was consistent with the formation of 2,3-diaminophenazine ($\lambda_{\text{max}} = 445$ nm, pH 5.0), a product of the oxidation of 2-phenylenediamine (36). The rate of its formation with YvrK was estimated to be $-0.1 \%$ that of oxalate decarboxylation (assuming $\varepsilon = 10,000 \text{ M}^{-1} \text{cm}^{-1}$ at 445 nm for the oxidation product).

Oxalate oxidase can be assayed in real time by coupling the production of $\text{H}_2\text{O}_2$ to the horseradish peroxidase-catalyzed oxidation of ABTS. This proved not to be practical with YvrK because of a slow rate of peroxidase-independent, oxide-dependent oxidation of ABTS. The spectrum of the product was consistent with the single-electron oxidation of ABTS (37). The rate of this reaction was 0.5% that of decarboxylation when comparing assays with an oxalate concentration equal to its $K_m$ for the decarboxylase reaction (15 mM). Therefore, the ability of YvrK to oxidize dyes was not restricted to 2-phenylenediamine.

YvrK produced $\text{H}_2\text{O}_2$ in the presence of oxalate, indicating that it possesses some oxalate oxidase activity also. The rate of oxalate oxidation relative to decarboxylation was 0.2% when comparing assays with an oxalate concentration that was saturating with respect to the decarboxylation reaction. The rate of oxalate oxidation, and therefore $\text{H}_2\text{O}_2$ production, was similar to the rate of ABTS oxidation. The possibility that the oxidation of ABTS was actually $\text{H}_2\text{O}_2$-dependent, rather than directly oxalate-dependent, was ruled out with control reactions in the presence and absence of oxalate and $\text{H}_2\text{O}_2$. YvrK does not therefore possess formal peroxidase activity. Because $\text{H}_2\text{O}_2$ production was significantly lower in the presence of ABTS, it is possible that the dye intercepts the oxalate oxidase side reaction. YvrK exhibited no superoxide dismutase activity at pH 7.2 and no phosphodiesterase activity at either pH 5.0 or 8.0.

Activities of YoaN and YxaG—Recombinant YoaN in crude cell-free extracts exhibited oxalate decarboxylase activity when Mn$^{2+}$ salts were present in the growth medium during expression. This is the first demonstration of an activity associated with YoaN. The addition of metal ions other than Mn$^{2+}$ did not support decarboxylase activity, indicating that YoaN, like YvrK, is manganese-dependent. Although the level of overexpression of YoaN appeared to be similar to that of YvrK, the decarboxylase activity of YoaN was typically half that of recombinant YvrK in cell-free extracts. YoaN also possessed small but routinely detectable oxalate-dependent ABTS and 2-phenylenediamine oxidation activities but only traces of oxalate oxidase activity and no superoxide dimituate or phosphodiesterase activities. Because crude extracts from cells that were not induced did not possess any of these activities, it would appear that YoaN, as predicted, is an oxalate decarboxylase with similar properties to YvrK. By contrast, recombinant YxaG in crude extracts did not possess any of the above activities whether or not manganese salts were added to the growth medium during expression.

Metal Analysis of YvrK—Metal analysis using inductively coupled plasma emission spectroscopy showed that the enzyme contained between 0.86 and 1.14 atoms of manganese per subunit depending on the batch. The data consistently show close to 1 and not 2 manganese atoms bound per subunit. Although samples that were exhaustively dialyzed gave the lowest levels of manganese, the majority of this metal ion remained tightly bound to the enzyme. The enzyme contained considerably less iron (0.35 atoms/subunit), copper (0.04–0.17), zinc (0.15–0.20), and cobalt (<0.08). All metals except iron were present in buffer controls at concentrations at least one order of magnitudate less than the protein subunit concentration in test solutions. Given the high background concentrations of iron, con-

![Fig. 3. Michaelis-Menten kinetics of oxalate decarboxylation by YvrK.](http://www.jbc.org/)

Each subunit bears one. The addition of dithiothreitol prevented dimerization from occurring.

Decarboxylase Activity of YvrK—The oxalate dependence of the oxalate decarboxylase activity of YvrK followed Michaelis-Menten kinetics (Fig. 3). The $K_m$ (15 mM) was higher than that reported for the Aspergillus niger (2–4 mM (32)), Myrothecium verrucaria (1.7 mM (33)), and F. velutipes (4.5 mM (34)) enzymes. The $V_{\text{max}}$ (75 $\mu$mol min$^{-1}$ mg$^{-1}$) corresponded to a $k_{\text{cat}}$ of 54 s$^{-1}$ and was lower than that reported for the F. velutipes enzyme (166 $\mu$mol min$^{-1}$ mg$^{-1}$ (34)). The addition of MnCl$_2$ (1.5–60 mM) to assay mixtures did not result in an increase in activity, and dialysis of the enzyme for 17 h at 6°C with buffers that were devoid of manganese salts did not lead to a detectable decrease in activity.

$O_2$ Dependence of YvrK Decarboxylase Activity—The wild-type bacterial enzyme is known to be less active when it has undergone vacuum-N$_2$ gas flush cycles and is then assayed in an atmosphere maintained at <1 ppm of O$_2$ (7). These experiments have been repeated with recombinant YvrK together with further measures to remove dissolved O$_2$. The addition of 1 mM dithionite to assay mixtures resulted in a complete loss of oxalate decarboxylase activity. Although this result was consistent with the O$_2$ dependence of the reaction, dithionite is a powerful single-electron reductant that could have affected the enzyme in other ways. Enzyme that had been treated with dithionite and exposed to atmospheric O$_2$ before assay regained only 18% of its activity, suggesting some irreversible effect of dithionite (or an effect of its oxidation product sulfate) on activity.

To remove dissolved O$_2$ without the complications posed by dithionite, the experiments were repeated in the presence of glucose oxidase and excess glucose. Catalase was also added in order to remove the H$_2$O$_2$ produced by glucose oxidase. YvrK retained only 20 and 16% of its activity using 4 and 8 mM glucose, respectively. This result confirmed the dependence of decarboxylation on O$_2$. Enzyme that was treated with this O$_2$-removing mixture and then exposed to excess atmospheric O$_2$ before assay regained 131% of its original activity. Assays containing catalase, but no glucose or glucose oxidase, gave 95% the original activity, indicating that the enzyme was not activated by the presence of catalase. Overall, these results clearly show the oxalate decarboxylase activity of YvrK to be O$_2$-dependent.

Minor Activities of YvrK—The oxalate decarboxylase assay method described by Magro et al. (30) recommends the addition of 2-phenylenediamine to protect the Sclerotinia sclerotiorum enzyme. Emiliani and Riera (35) noted that when assaying the A. niger enzyme in the presence of this compound, the solution became pale yellow in color. We have observed a small but significant protective effect of this compound on YvrK decarboxylase activity during turnover, as well as the formation of a pale yellow color. The spectrum of the new compound was consistent with the formation of 2,3-diaminophenazine ($\lambda_{\text{max}} = 445$ nm, pH 5.0), a product of the oxidation of 2-phenylenediamine (36). The rate of its formation with YvrK was estimated to be $-0.1 \%$ that of oxalate decarboxylation (assuming $\varepsilon = 10,000 \text{ M}^{-1} \text{cm}^{-1}$ at 445 nm for the oxidation product).
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Fig. 4. UV-visible spectrum of YvrK. The protein (10 μM) in 50 mM Tris buffer, pH 7.0, containing 100 mM NaCl and 10 μM MnCl₂, has a \( \lambda_{\text{max}} \) of 278 nm and a weak shoulder extending beyond 300 nm. Similar spectra were observed in the absence of salts.

Fig. 5. X-band EPR spectrum of YvrK. The EPR spectrum of dialyzed oxalate decarboxylase (480 μM) in 50 mM Tris buffer, pH 7.0, at 20 K shows a six-line hyperfine Mn(II) spectrum at \( g = 1.996 \) and a smaller signal at \( g = 4.1–4.3 \).

fidem in the precise value for iron content in YvrK was less than for the other metal ions.

UV-visible Spectrum of YvrK—The UV-visible spectrum of purified YvrK showed no characteristic chromophores associated with organic or metal ion cofactors (Fig. 4). Although it is an essentially normal spectrum for a protein (\( \lambda_{\text{max}} \) 278 nm), there is a weak shoulder that extends beyond 300 nm to longer wavelengths. This gives YvrK solutions a pale yellow color at high concentrations. A similar shoulder has also been observed with the Mn(II)-containing enzymes oxalate oxidase (15) and manganese lipoxygenase (38). This weak shoulder does not seem to add significantly to the absorbance at 278 nm because its intensity is slightly lower, rather than higher, than that predicted from two independent protein assays.

EPR Spectroscopy of YvrK—The oxidation state of the manganese in the enzyme was determined using EPR spectroscopy (Fig. 5). The six-line signal centered around \( g = 1.996 \) clearly showed the presence of a Mn(II) S = 5/2 spin system in an axial environment with zero field splitting much less than the Zeeman splitting. The hyperfine structure of the Mn(II) signal was quite different from that of the MnCl₂, indicating that the Mn(II) was protein-bound, and it was more complex than that of oxalate oxidase (15). The hyperfine coupling constant, \( A \), was 9.5 mT. Integration of the spectrum using a MnCl₂ standard under identical conditions indicated the presence of between 50 and 80% Mn(II), using the metal analysis data as the 100% reference. The addition of dithionite to the enzyme resulted in an increase in the Mn(II) signal intensity such that 100% of the enzyme appeared to be in the Mn(II) oxidation state. Exposure of the dithionite-reduced enzyme to atmospheric oxygen over a period of hours resulted in reoxidation of about 20% of the metal ion, presumably to EPR silent Mn(III). Samples that were boiled and reduced with dithionite exhibited spectra typical of Mn(II) in buffered solution. Integration of these spectra gave values essentially identical to those expected from the metal analysis data. The intensity and line shape of the Mn(II) signal of as-isolated enzyme were not significantly affected by flushing the sample with gaseous oxygen at 23 °C over a period of hours. These results are consistent with the enzyme being isolated in predominantly but not exclusively the Mn(II) oxidation state.

An unusual feature of the YvrK spectrum was the presence of a signal at \( g = 4.1–4.3 \), which had a hyperfine coupling constant of 8.0–8.5 mT. A similar feature has also been observed with oxalate oxidase (15). It disappeared after YvrK was boiled, suggesting that it arises from protein-bound Mn(II). The intensity of the low field signal was similar when 10% glycerol was present, indicating that it was not an artifact resulting from association during the freezing of the sample. Furthermore, in samples where association has occurred, half-field signals (\( \Delta M_I = \pm 2 \)) at \( g = 4 \) are observed together with the usual \( g = 2 \) signal (\( \Delta M_I = \pm 1 \)), where the relative intensity of the half-field transition is typically 100-fold lower. However, the relative intensity of the \( g = 4 \) signal observed here is about 10-fold lower. In addition, the hyperfine splitting parameters of both transitions should be identical. Therefore, the additional low field feature indicates the presence of some Mn(II) (\( \approx 8\% \)) that is in a different environment. Such isotropic and rhombic signals at \( g = 4 \) from Mn(II) complexes are typically associated with tetrahedral or highly distorted octahedral environments with substantial zero field splitting that is greater than the Zeeman splitting (39). There was no indication of any other EPR active metal ion, including Fe(III), or organic free radical before or after reduction or boiling.

DISCUSSION

General Considerations—YvrK and YoaN are the first oxalate-degrading enzymes to be overexpressed in a soluble and active form in a microbial host. This was possible only when the E. coli cells were heat-shocked prior to overexpression, presumably leading to the production of chaperones that facilitated the correct folding of the recombinant enzymes. The inclusion of a manganese salt in the growth medium during expression was also essential, indicating that the supply of manganese from Luria-Bertani broth via an endogenous manganese transport system is insufficient for expression of these Mn-proteins in E. coli. Perhaps a high extracellular Mn²⁺ ion concentration enables it to enter cells along the Mg²⁺ transport system.

It is clear from the expression requirements, metal analysis, and EPR spectroscopy that YvrK, like oxalate oxidase (15), is a manganese-containing enzyme as predicted (7). This metal ion appears to bind tightly to the enzyme, since exhaustive dialysis did not result in significant loss of manganese content or activity. YvrK is isolated predominantly in the Mn(II) oxidation state. It is possible that it is entirely in the Mn(II) oxidation state in the reducing environment of the cytoplasm of B. subtilis, because the enzyme was purified under aerobic conditions in the absence of reductants. Additional studies are required to address the redox properties of the enzyme. We have also shown that YoaN is another oxalate decarboxylase that, from its expression requirements, appears to be a Mn-enzyme also, as previously predicted (4, 7). What is also clear is that YvrK is O₂-dependent. It is the dependence of catalysis on O₂ that strongly implicates manganese in a free radical catalytic mechanism. Because dioxygen dependence has also been reported for the M. verrucaria, A. niger, and F. velutipes enzymes (32, 33, 40), it would seem highly likely that these also contain manganese.

Although many germin-like proteins are emerging from genome sequencing projects (10), few have known catalytic activities. Those that have been defined include manganese-depend-
ent superoxide dismutases (17, 18) and nucleotide-sugar pyrophosphatase/phosphodiesterases (19). Of interest was whether the three bacterial bicupins presently described possessed any of these two activities, but none were detected. In addition, YxaG did not possess any oxalate-degrading activity. It is therefore not clear what, if any, reaction YxaG catalyzes. It is interesting to note that YxaG could be overexpressed in a soluble form without the addition of supplemental metal ion salts to the growth medium, suggesting that it may not be a metalloprotein. However, it is possible that either the apoprotein is soluble in this case or that another more abundant metal ion can substitute for manganese. The finding that both YvrK and YoaN are oxalate decarboxylases, and that YxaG is not, is consistent with the way in which they cluster in recent phylogenetic analyses (10).

Although we have shown that YvrK catalyzes several side-reactions, it is primarily an oxalate decarboxylase. We have previously shown that it is expressed in B. subtilis specifically when the organism is exposed to acid pH stress, suggesting a role in proton consumption within the cytoplasm (7). In the present study we have shown that YoaN is an oxalate decarboxylase with properties similar to YvrK. However, it remains to be seen under what physiological conditions YvrK is expressed in B. subtilis and what role it may have. It is proposed that yvrK be renamed oxdC, consistent with the oxalate decarboxylase gene from the white rot wood-decaying fungus F. velutipes (34). Because we have also shown yoaN to code for an oxalate decarboxylase, it is proposed that this be renamed oxdD. Although we have shown that YxaG can be expressed as a soluble protein in E. coli, and is therefore no longer just a hypothetical open reading frame, its function remains obscure.

Structure of YvrK—The main g = 2 Mn(II) EPR signal of YvrK exhibited a hyperfine coupling constant, A, of 9.5 mT. Values of about 8–10 mT are typical for a Mn(II) ion in either a penta- or hexacoordination environment with oxygen or nitrogen ligands making bonds of weak covalency with the metal ion (41). This value compares well with those of other hexacoordinate Mn(II) enzymes with distorted octahedral geometries such as oxalate oxidase (–9 mT (15)), concanavalin A (9.3 mT (42)), manganese catechol dioxygenase (9.5 mT (43)), and manganese lipoxygenase (9.5 mT (44), assuming an active site geometry like the iron lipoxygenase (45)). By contrast, the minor EPR signal of YvrK was at g = 4 and had a smaller hyperfine coupling constant of 8.0–8.5 mT. Similar low field signals have been reported for the pentacoordinate Mn(II) of the reduced forms of both wild-type (46) and mutant (47) mitochondrial Mn-superoxide dismutases (A = 8.6 mT). These data suggest that the two EPR signals of YvrK arise from Mn(II) ions in different environments: an abundant hexacoordinate Mn(II) with small zero field-splitting parameters and a less abundant pentacoordinate Mn(II) with larger zero field-splitting parameters.

The main EPR signal of YvrK is consistent with an active site resembling that of oxalate oxidase (Fig. 2). Modeling of the N- and C-terminal domains of YvrK, based on homology with oxalate oxidase, showed that it is possible for both domains to form β-barrels with the Glu and three His residues of the conserved motif in each domain clustered in their interiors (not shown). On this basis alone, it would be predicted that the enzyme had the potential for each subunit to bind 2 manganese ions and for both domains to be catalytically active. However, metal analysis and integration of the Mn(II) EPR spectrum of samples after boiling and reduction showed that each subunit binds only a single Mn(II) ion. In addition, incubation of the purified enzyme with a Mn2+ salt did not lead to an increase in enzyme activity. Therefore only one site can be fully occupied by manganese. Perhaps the small g = 4 signal in the EPR spectrum arises from partial occupancy of the second site. Importantly, there do not appear to be two similar but non-identical sites with overlapping signals. The second potential active site could be occupied by a metal ion(s) other than a manganese ion. Partial occupancy by EPR silent Fe(II) is possible, because iron was detected by metal analysis at up to 0.35 atoms/subunit. Significantly less copper, zinc, and cobalt were detected, ruling out the presence of these metals.

The question arises as to which of the two potential active sites is functional. Both gel filtration and nondenaturing polyacrylamide gel electrophoresis of YvrK showed it to be a hexamer in solution. Crystal structures of two distantly related bicupins that can form hexamers in solution have been solved; hexameric homogentisate 1,2-dioxygenase (24) and trimeric proglycinin (13), which can form hexamers after proteolytic processing to form glycinin. Both are thought to oligomerize as dimers of trimers with the same geometric arrangement. When homology models of the oxalate decarboxylase subunit, based on homology with canavalin and phaseolin, are used to generate a model of the hexamer, based on homology with the hexameric bicupins above, only the N-terminal domain active sites are solvent accessible (not shown). The potential C-terminal domain active site entrances are predicted to be buried within the interdimer interface. It is interesting that it has been suggested that the bicupins YvrK and YoaN evolved through the gene duplication of a common monocupin ancestor, YkrZ (4). It was also noted that their C-terminal domains show the highest degree of similarity with YkrZ; this is consistent with the likely hexameric structure, because there would be selective pressure to maintain stable core structures. Structural and mutagenesis studies will be required to confirm these hypotheses.
Proposed Decarboxylase Catalytic Cycle—Emiliandi and Riera (35) recognized many years ago that the O₂ requirement and small oxidase activity of oxalate decarboxylase suggested free radical chemistry. However, because they were not aware of the presence of manganese, they proposed an unusual aromatic amino acid–O₂ adduct to be the active cofactor, for which we have observed no evidence using EPR spectroscopy. We have previously proposed a catalytic mechanism involving Mn(II) for oxalate oxidation (15). The requirement of O₂ and manganese for both the oxidation and decarboxylation of oxalate, coupled with the likely similarity of the active sites of these enzymes, suggests similar catalytic cycles.

Although the enzyme is isolated in predominantly the Mn(II) oxidation state, it is not clear what the oxidation state of the active resting state of the enzyme is. It could either be Mn(II), Mn(III)-superoxo (Mn(II) with O₂ bound), or Mn(III). The failure of glucose oxidase to completely inhibit the enzyme in anaerobic conditions suggests that some O₂ could have remained tightly bound to YvrK. This observation would favor the Mn(III)-superoxo form as the active species. Although a superoxy signal would be expected in the EPR spectrum, the signal may have been too small and broad to be observed clearly along with the dominant Mn(II) signal.

In any case, let us first consider catalytic cycles that formally begin with Mn(II). Both oxalate and O₂ could bind to give a Mn(III)-superoxo-oxalato species (Fig. 6). Whatever the order of binding of the substrates, the decarboxylation of oxalate would lead to the formation of a Mn(II)-bound formyl radical species that could combine with the superoxy to form a Mn(II)-percarbonate intermediate. It is from this point that the decarboxylase and oxidase reactions could diverge. The loss of formate coupled with protonation at the carbon atom would lead to a Mn(III)-superoxo species that would be in equilibrium with the Mn(II) form and free O₂. It is the combination of Mn(II) with O₂ that provides a two-electron sink for the initial decarboxylation of oxalate. By contrast, other decarboxylases use thiamine diphosphate or pyridoxal phosphate for such a two-electron sink. The precise delivery of a proton to a carbon atom to produce formate is crucial in the decarboxylase reaction (and this could perhaps occur just prior to the formation of the C–O bond of percarbonate). On the other hand, the oxidase reaction would result from the loss of CO₂ and H₂O₂ from percarbonate coupled with protonation at oxygen. Alternatively, nucleophilic attack of the carbonyl carbon of percarbonate by a hydroxide ion would give the oxidase reaction via carbonate. This alternative route would not necessarily be on-enzyme because percarbonate would hydrolyze spontaneously in water.

Mn(III)-based cycles would be very similar overall (Fig. 6). There is chemical precedence for some of the partial reactions (48), as discussed previously (15). However, a key aspect of this cycle is the requirement for the loss of one molecule of CO₂ before O₂ could participate; this makes these cycles less attractive than the Mn(II)-based cycles because only a single-electron sink would be in place for the crucial initial decarboxylation step. Furthermore, the involvement of O₂ in the Mn(III)-based decarboxylase reaction would seem to be superfluous because the catalytic cycle could be completed without it.

An alignment of the motifs of several cupins (Fig. 1) shows that Arg⁸² and Glu⁹⁰ of YvrK represent the only general acids or bases that are uniquely conserved in this region among the oxalate decarboxylases. Homology models of YvrK show that one or the other of these residues, depending on the precise structural alignment used, could lie close to the manganese and be responsible for proton donation to carbon as described above. Further studies are required to identify whether one of these residues, another amino acid, or a water molecule performs this function. Consistent with the proposed divergent mechanisms was the observation that YvrK and the A. niger enzyme (35) are capable of a small amount of oxalate oxidase activity, which may reflect a lack of complete control over proton delivery. Importantly, O₂ functions as a cofactor in the proposed mechanism for oxalate decarboxylase, a unique role for O₂ in biology.

The exact details of the oxalate decarboxylase-catalyzed oxidation of dyes will need further investigation. The rate of this reaction was less than 1% that of oxalate decarboxylation by YvrK, and it is possible that the dyes intercept the slow oxalate oxidation side reaction. Perhaps the dyes protect the enzyme’s activity by preventing autoxidation. Nevertheless, the single-electron oxidation of ABTS provides additional evidence for the capability of this enzyme to catalyze free radical reactions. It is interesting to note that some manganese-dependent oxy- genases are known (41), but none so far are known to belong to the cupin superfamily.

We have overexpressed the first oxalate decarboxylase and shown that it contains 1 manganese ion/subunit. This observation provides an insight into how the enzyme utilizes O₂ in a catalytic cycle that probably involves free radical intermediates. We are currently using a combination of structural and biochemical approaches to identify the active site, the redox properties of the active enzyme, and how it controls catalysis such that oxalate is decarboxylated rather than oxidized.

Acknowledgments—We thank Drs. Laura Requena, Simon J. George, and David J. Lowe for helpful discussions, particularly regarding EPR spectroscopy, and Dr. Simon Foster, University of Sheffield, UK, for the gift of B. subtilis.
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Oxalate Decarboxylase Requires Manganese and Dioxygen for Activity: OVEREXPRESSON AND CHARACTERIZATION OF BACILLUS SUBTILIS YvrK AND YoaN

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J. Biol. Chem. 2001, 276:43627-43634.
doi: 10.1074/jbc.M107202200 originally published online August 23, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M107202200

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