A Closed Conformation of *Bacillus subtilis* Oxalate Decarboxylase OxdC Provides Evidence for the True Identity of the Active Site*

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Oxalate decarboxylase (EC 4.1.1.2) catalyzes the conversion of oxalate to formate and carbon dioxide and utilizes dioxygen as a cofactor. By contrast, the evolutionarily related oxalate oxidase (EC 1.2.3.4) converts oxalate and dioxygen to carbon dioxide and hydrogen peroxide. Divergent free radical catalytic mechanisms have been proposed for these enzymes that involve the requirement of an active site proton donor in the decarboxylase but not the oxidase reaction. The oxidase possesses only one domain and manganese binding site per subunit, while the decarboxylase has two domains and two manganese sites per subunit. A structure of the decarboxylase together with a limited mutagenesis study has recently been interpreted as evidence that the C-terminal domain manganese binding site (site 2) is the catalytic site and that Glu-333 is the crucial proton donor (Anand, R., Dorrestein, P. C., Kinsland, C., Begley, T. P., and Ealick, S. E. (2002) *Biochemistry* 41, 7659–7669). The N-terminal binding site (site 1) of this structure is solvent-exposed (open) and lacks a suitable proton donor for the decarboxylase reaction. We report a new structure of the decarboxylase that shows a loop containing a 3₁₀ helix near site 1 in an alternative conformation. This loop adopts a “closed” conformation forming a lid covering the entrance to site 1. This conformational change brings Glu-162 close to the manganese ion, making it a new candidate for the crucial proton donor. Site-directed mutagenesis of equivalent residues in each domain provided evidence that Glu-162 is the central residue in both the oxidase and decarboxylase reaction. This loop mutation of Glu-162 to Asp creates a new site for Mn(II), while Glu-162 to Lys drastically reduces Mn(II) affinity (15). Two structures of the decarboxylase domain with Glu-162 and Lys-162 have been solved with and without Mn(II), respectively. OxdC has been shown to contain manganese ions in predominantly the Mn(II) oxidation state (14), and it is a member of the cupin superfamily. Oxalate decarboxylase (EC 4.1.1.2) from barley shares many properties with the decarboxylases: it contains manganese in predominantly the Mn(II) oxidation state (12). The oxalate decarboxylase sequences show that they belong to the cupin superfamily that is characterized by conserved motifs and a β-barrel domain fold (13). They are in fact members of a subset of this family, the bicupins, because the cupin domain occurs twice, presumably due to an evolutionary gene duplication event.

Oxalate decarboxylase (EC 4.1.1.2) catalyzes the conversion of oxalate to carbon dioxide and formate. Until recently, the best characterized enzymes have been from fungi (7). The extracellular fungal enzymes are induced by acid pH and are thought to control excess oxalic acid levels (8–10). It is likely that the decarboxylase is involved in the elevation of external pH because the reaction involves the consumption of a proton. The first bacterial oxalate decarboxylase was recently identified in *Bacillus subtilis* (OxdC, formerly known as YoaN) (11). This cytoplasmic enzyme was induced by low pH and not by oxalate salts suggesting that it is involved in the elevation of cytoplasmic pH. *B. subtilis* was shown to possess a second oxalate decarboxylase because a hypothetical protein homologous to OxdC exhibited oxalate decarboxylase activity when expressed in *Escherichia coli* (OxdD, formerly known as YoaN) (12). The oxalate decarboxylase sequences show that they belong to the cupin superfamily that is characterized by conserved motifs and a β-barrel domain fold (13). They are in fact members of a subset of this family, the bicupins, because the cupin domain occurs twice, presumably due to an evolutionary gene duplication event.

*B. subtilis* OxdC has been shown to contain manganese ions in predominantly the Mn(II) oxidation state (12). It was also shown that catalysis was uniquely dioxygen-dependent despite the reaction involving no net redox change. The oxalate oxidase (EC 1.2.3.4) from barley shares many properties with the decarboxylases: it contains manganese in predominantly the Mn(II) oxidation state (14), and it is a member of the cupin superfamily. However, unlike the decarboxylases, the oxidase is a monocupin (15) that converts oxalate and dioxygen to carbon dioxide and hydrogen peroxide in a net redox reaction using dioxygen as a substrate.
The lack of any other acidic or basic amino acid residue in the residues of the cupin motif (22) as predicted previously (14) showed the manganese ion to be coordinated by a Glu and 3 His specificities particularly in terms of the identity of the crucial deca-
boxylase and oxidase would provide a simple explanation expected in the oxalate-degrading enzymes.

Given the common properties of the oxalate decarboxylases and oxidases, we have proposed a divergent catalytic mechanism for these enzymes (12). An important feature of this mechanism is the binding of oxalate and dioxygen to the Mn(II) ion to give a Mn(III)-superoxo species (Fig. 1). This acts as an electron sink to facilitate the decarboxylation of oxalate to give a manganese ion-bound formyl radical anion intermediate. Importantly what differentiates the two enzymes is the ability of the decarboxylase to specifically protonate the carbon atom of this intermediate.

Evidence to support these mechanisms comes from electrochemical studies that show that oxalate radicals rapidly undergo decarboxylation (16). In addition, recombinant plant oxalate oxidase has been shown to produce formyl radicals on addition of oxalate using radical spin traps (17), indicating that the oxidase is capable of radical chemistry. More recently, heavy atom isotope effects with the bacterial decarboxylase have provided the first direct evidence for a reversible, proton-coupled, electron transfer from bound oxalate to the manganese ion to give an oxalate radical that decarboxylates to give a formate radical (18). Furthermore this study also shows that the carboxylate group of the oxalate that goes on to form formate bears the radical and has a low bond order as shown in Fig. 1. There are several examples of inorganic compounds that show percarbonate bound in a bidentate mode to the metal ions of platinum, palladium, nickel, and rhodium (19–21) analogous to that proposed for oxalate oxidase. There is also an interesting new structure of a manganese-containing hypothetical protein (Tm1287) of the cupin superfamily from Thermotoga maritima that has oxalate bound in a bidentate mode to the manganese ion (Protein Data Bank entry 1O4T) rather than the monodentate mode expected in the oxalate-degrading enzymes.

It was anticipated that the structures of both the oxalate decarboxylase and oxidase would provide a simple explanation of how the active sites of these enzymes control their reaction specificities particularly in terms of the identity of the crucial proton donor in the decarboxylase. The oxidase structure showed the manganese ion to be coordinated by a Glu and 3 His residues of the cupin motif (22) as predicted previously (14). The lack of any other acidic or basic amino acid residue in the active site of the oxidase is consistent with the mechanistic hypothesis. A structure of the bacterial decarboxylase confirmed the prediction that this bicupin was composed of two β-barrel cupin domains per subunit in a hexameric arrangement (23). Both domains contained manganese ions with the same amino acid coordination environment as the oxidase. The question then arose as to which sites are catalytically active. Anand et al. (23) had proposed that only the C-terminal domain site (site 2) is the active one. This was based on the observation that only this site has an acidic group (Glu-333) close to a manganese ion that would be capable of protonating the formyl radical intermediate at the optimum pH of the enzyme of between 3.5 and 5.0 (11, 18). This hypothesis was supported by the 25- and 4-fold lowering of activity of an E333A mutant according to assays detecting the production of formate and carbon dioxide, respectively.

This report describes a new B. subtilis oxalate decarboxylase OxdC structure with an altered conformation. This provides evidence that Glu-162, rather than Glu-333, is the crucial active site proton donor required for catalysis and that the N-terminal manganese binding site (site 1), rather than site 2, is catalytically active. Site-directed mutagenesis studies supporting these conclusions are also described.

EXPERIMENTAL PROCEDURES

Materials—All materials and biochemicals were of the highest grade available and, unless stated otherwise, were purchased from Sigma. Protein concentration was determined using the Pierce Coomassie Plus 200 assay. Horseradish peroxidase (HRP4C) was purchased from Biozyme Laboratories Ltd. (Blaoenavon, Gwent, Wales, UK).

Expression, Purification, and Crystallization of Non-His-tagged OxdC—B. subtilis OxdC was cloned, expressed, and purified as described previously (12). Protein expression was induced in E. coli using isopropyl-β-D-thiogalactopyranoside following the addition of 5 mM MnCl₂ to the growth medium and after heat shocking the culture at 42 °C for 2 min. The protein was purified using anion exchange and gel filtration chromatography and was concentrated to 9 mg ml⁻¹ by ammonium sulfate precipitation. The protein was buffer-exchanged into 20 mM Tris, pH 7.0, and filtered through a 0.1-μm Ultrafilter filter (Millipore) prior to crystallization. Crystals were grown by hanging drop vapor diffusion using VDX plates (Hampton Research) at 18 °C, and drops consisted of 1 μl of protein mixed with 1 μl of precipitant. Crystallization conditions for the recombinant, non-His-tagged B. subtilis OxdC were derived independently of the conditions previously reported for the His-tagged protein (23). Nevertheless the final optimized conditions of 8% polyethylene glycol 8000 in 100 mM Tris, pH 8.5, at 18 °C were very similar to those published.

X-ray Crystallography—All crystal manipulations were performed using Hampton Research tools. The crystals were cryoprotected using an artificial mother liquor containing 25% ethylene glycol in place of the buffer volume and flash-cooled to 100 K in a stream of gaseous nitrogen using an X-Stream cryocooler (Rigaku/MSC). Diffraction data to 2.0-Å resolution were collected in-house using a Mar 345 image plate detector (x-ray research) mounted on a Rigaku RU-H3RHB rotating anode x-ray generator (operated at 50 kV and 100 mA) fitted with Osram confocal optics and a copper target (CuKα; λ = 1.542 Å). X-ray data were processed using the HKL software package (24). All other data collection and processing statistics were carried out using programs from the CCP4 software suite (25). The crystals were essentially isomorphous with those obtained previously (23). They belonged to space group R32 with cell parameters of a = b = 154.7 Å, c = 122.8 Å (hexagonal setting) and contained a single 43.6-kDa monomer per asymmetric unit, giving an estimated solvent content of 62% (26).

Fourier electron density maps. Positional and thermal parameters of the model were subsequently refined using REFMAC5. A subset of the data comprising a random 5% of the reflections was excluded from refinement and used in the calculation of the “free” (R_free) crys-
B. subtilis Oxalate Decarboxylase

Oxalate Decarboxylase Assay—One unit of enzyme activity was defined as the conversion of 1 μmol of substrate to product/min. Oxalate decarboxylase activity at pH 4.0 was determined using a stopped assay at 26 °C where the production of formate was coupled to the reduction of NAD by formate dehydrogenase as described previously (12). Unless otherwise stated, the oxalate decarboxylase reaction mixtures contained 150 mM potassium oxalate, 100 mM sodium citrate, pH 4.0, 300 μM α-phenylethylamine, 10 μM bovine serum albumin, and enzyme (up to 0.9 mg ml⁻¹) and were incubated for 10 min.

Oxalate Oxidase and Dye Oxidation Assays—Oxalate oxidase activity was determined spectrophotometrically at 23 °C using a continuous assay in which the production of hydrogen peroxide was coupled to the oxidation of ABTS¹ using horseradish peroxidase as described previously (14). Reaction mixtures contained 50 mM sodium citrate buffer, pH 4.0; horseradish peroxidase (25 units, as defined by supplier), 5 mM ABTS, 20 mM potassium oxalate, and enzyme (up to 0.02 mg ml⁻¹). To distinguish between oxalate oxidase (i.e., hydrogen peroxide production) and direct oxalate-dependent dye oxidation activities of the enzyme, controls without peroxidase were necessary.

Molecular Modeling—Molecular modeling calculations were performed with Insight II software (release 2000.1, Accelrys Ltd., Cambridge, UK) using the Discover module and consistent valence force field. All water molecules were omitted. The geometries of the oxalate and O₂ ligands were constructed using geometric parameters from known crystal structures, and partial atomic charges were estimated based on the values for related species. Since Discover contains a potential for iron but not manganese, the former was used to substitute for the manganese atom. The following constraints were set for the geometry optimization: residues 6–81 and 202–382, all atoms fixed; residues 81–160 and 160–201, backbone atoms only fixed. Residues 161–165 were fully unrestrained. The oxalate, O₂, and manganese were all kept fixed. The optimized geometry was validated using the Procheck module of Insight II.

RESULTS

General Comparison of the OxdC Structures—The first study describing the structure of B. subtilis OxdC presented two models determined at resolutions of 1.75 Å (Protein Data Bank entry 1J58) and 1.9 Å (Protein Data Bank entry 1L3J) that were derived from crystals that were grown in the absence and presence of 10 mM formate, respectively (23). These two structures were essentially identical, both having a formate molecule bound to manganese binding site 1 whether formate was added or not. The main structural differences in the formate-soaked structure are the presence of a second, loosely bound, formate molecule on the protein surface and the lack of one water molecule coordinated to the manganese in binding site 1. These differences are unlikely to have any functional significance. Therefore, we used the higher resolution, non-formate-soaked crystal structure (Protein Data Bank entry 1J58) for comparisons with our model.

The full sequence of OxdC (GenBank™ accession number 21362729) contains 385 amino acids. In our model of the structure, it was possible to resolve residues 6–382 with confidence. Thus, 5 additional amino acids were included at the termini compared with the previously published structures that comprised residues 8–379 only. In addition, a Tris molecule was clearly resolved at each subunit interface in the central solvent channel along the 3-fold axis of the hexamer. However, there was no evidence of the formate molecules nor of a surface-bound metal ion (presumed to be a magnesium ion) observed in the previous structures.

Conformational Changes in OxdC—Not surprisingly, the coordinates of our OxdC structure superimpose closely with those of the previously published structure (23), giving a root mean square deviation (r.m.s.d.) of 0.52 Å based on all common main-chain atoms. Nevertheless, aside from a number of altered surface side-chain conformations, there are two regions

| Table I Summary of x-ray data and model parameters for OxdC |
|-------------------------------------------------------------|
| Data collection                                            |
| Resolution range (Å)                                       |
| 40–2.0 (2.03–2.00)                                         |
| Unique reflections                                        |
| 35,069                                                    |
| Completeness (%)                                          |
| 100.0 (99.8)                                               |
| Redundancy                                                |
| 23.6                                                      |
| Rmerge (based on 95% of data) (%)                          |
| 12.7                                                     |
| Rmerge (based on 5% of data) (%)                          |
| 15.8                                                     |
| DPF (based on Rmerge) (Å)                                  |
| 0.999                                                    |
| Residuals with most favored Ψ/Φ (%)                       |
| 58.6                                                     |
| r.m.s.d. bond distances (Å)                                |
| 0.013                                                    |
| r.m.s.d. bond angles (%)                                   |
| 1.409                                                    |
| Contents of model (molecules/non-hydrogen atoms)           |
| Protein (residues/atoms)                                  |
| 377/3,000                                                 |
| Manganese ions                                            |
| 2                                                        |
| Water                                                     |
| 402                                                      |
| Tris                                                      |
| 1/8                                                       |
| Average temperature factors (Å²)                           |
| 17.7                                                     |
| Main-chain atoms                                          |
| 19.7                                                     |
| Side-chain atoms                                          |
| 16.1                                                     |
| Manganese ions                                            |
| 29.9                                                     |
| Waters                                                    |
| 24.2                                                     |
| Overall                                                   |
| 16.6                                                     |
| r.m.s.d. es. vs. open structure (Protein Data Bank entry 1J58) |
| 0.53                                                     |

¹ The figures in parentheses indicate the values for outer resolution shell.
² Rmerge = Σ(|I-Ij|)/ΣIj, where Ij is the intensity of an observation of reflection j and I is the average intensity for reflection j. The R-factors Rmerge and Rfree are calculated as follows: r = Σ(|Iobs-Icalc|)/ΣIobs × 100 where Iobs and Icalc are the observed and calculated structure factor amplitudes, respectively.
³ Diffraction-component precision index (34); an estimate of the overall coordinate errors were calculated using the REFMAC5 (27).
⁴ As calculated using PROCHECK (35).
⁵ After least-squares superposition based on all common main-chain atoms.
where the structures were noticeably different. A minor change occurs in a surface region and is largely restricted to residues 307 and 308. Despite the Cα atoms remaining in roughly the same positions, their backbone conformations are flipped between the two structures. The remoteness of this region to the manganese binding sites of our structure of OxdC are very similar in many respects to those already published (23). For example, each manganese ion is coordinated by 1 Glu and 3 His residues in an octahedral environment (Fig. 4). In addition, an Arg residue in an essentially identical arrangement is found nearby each metal ion. There are, however, some important differences. The manganese ion of site 1 in the closed structure has two water molecules bound (Fig. 4A) rather than a water molecule and a formate molecule in the open structure (Fig. 4C). The solvent-inaccessible cavity that is formed when the lid closes is occupied by these two water molecules together with a third water molecule (Figs. 2B and 4A). A critical change in site 1 of the closed structure is the movement of Glu-162 toward the manganese ion such that the side chain forms hydrogen bonds with this third water molecule and one of the water molecules that is directly bonded to the manganese. The Oe-2 atom of Glu-162 occupies essentially the same position as the distal oxygen atom of the formate that is bound to the metal ion in the open structure. This position is clearly predisposed to bind carboxyl groups favorably. In the open position, Glu-162 makes hydrogen bonding interactions with residues Thr-44 and His-299 from one neighboring subunit and Asp-297 from another adjoining subunit. The manganese binding site 2 is, by contrast, essentially identical in both structures (Fig. 4, C and D). The only difference is in the number of water molecules coordinated to the metal ion: one in the closed structure (Fig. 4B) and two in the open structure (Fig. 4D).

**Activities of OxdC Mutants**—To explore whether only one or both manganese binding sites possess oxalate decarboxylase activity, equivalent site-specific mutations were made in each site. An E333A mutant in site 2 has previously been reported to
have 4% the activity at pH 4.0 of the wild type enzyme using an assay to detect formate production (23). In the current study, this mutant had essentially the same relative activity (6%, Table III), but it also had a lower $K_m$ for oxalate giving a $k_{cat}/K_m$ that was 25% that of wild type. The E333Q mutant had no detectable activity, indicating that this mutation was significantly more disruptive. The equivalent mutations in site 1 gave an inactive enzyme with the E162A mutant and an E162Q mutant with a very low relative activity, indicating that this mutation was significantly more disruptive. The equivalent mutations in site 1 gave an inactive R92A mutant and a R92K mutant that had a low relative $k_{cat}/K_m$ (7%).

The value of $V_{max}$ for the oxalate decarboxylase activity of the wild type enzyme at pH 5.0 was similar to that obtained at pH 4.0 (the default pH for all of the enzyme assays). However, all eight mutant enzymes had <0.1% the activity of the wild type enzyme at pH 5.0 with little to distinguish between them.

The wild type non-His-tagged enzyme is known to possess small amounts of oxalate oxidase and oxalate-dependent dye oxidation activities (0.2 and 0.5% relative to oxalate decarboxylase activity, respectively) (12). Although these activities are small and difficult to detect, similar observations have also been made in this study with the His-tagged enzyme. Five of the eight mutants lost the oxalate oxidase side activity completely with only the E162A, E162Q, and E333A mutants retaining this activity according to the production of hydrogen peroxide. Six of the eight mutants had lower levels of dye oxidation activity compared with the wild type enzyme, but the E162A mutant had an essentially unchanged activity, and E162Q had more than double the activity.

Molecular Modeling—Monoprotonated oxalate and dioxygen were modeled into site 1 of the closed structure of OxdC. The first model had oxalate coordinated to the manganese ion in a monodentate mode via its carboxylate group to the site occupied by formate in the open structure. Dioxygen was placed in the remaining site occupied by water in the open structure.

The orientations of the oxalate and dioxygen were manually adjusted to minimize steric clashes with the amino acids lining the active site cavity, excluding those of the lid. The oxalate, but not the dioxygen, did clash a little with some side chains, most particularly with that of Glu-162. The conformations of the amino acid side chains were then energy-minimized while keeping the backbone atoms fixed. The resultant changes were largely restricted to the lid. A second round of energy minimization was required, with the backbone of the lid also free to move, to resolve the remaining steric clashes.

The resulting model showed some interesting features. The position of the lid in the final model (Fig. 4E) was a little more open than in the closed crystal structure (Fig. 4A). The Glu-162 side chain moved the most to allow room for the distal carboxylic acid group of the bound oxalate; the Glu-162 Ca and Oe-2 atoms moved about 2 and 4 Å, respectively. Despite these movements, there remained no solvent access to the site in the model. The proximal carbonyl group of oxalate was able to form hydrogen bonding interactions with both the Ne and Nε-2 atoms of Arg-92 with negligible change in the Arg side-chain conformation. These interactions resembled those between formate and Arg-92 in the open structure (Fig. 4C). The distal carboxylic acid group of oxalate was in a position to form favorable interactions with both Glu-162 and Tyr-200 in the model (Fig. 4E).

Modeling of oxalate and dioxygen with their coordination positions to the manganese ion reversed showed that while it may be possible for the active site to accommodate such an orientation, there were no potential stabilizing hydrogen bonding interactions. In addition, particularly unfavorable interactions between oxalate and Ile-114 led to the movement of this side chain (Cβ moved about 2.5 Å) and those around it in the hydrophobic core of the protein. There was no need for the lid to change conformation with this orientation. The cavity of site 2, on the other hand, did not allow room for both oxalate and dioxygen whichever orientation they adopted. Molecular modeling of site 2 with both ligands bound was not explored further because substantial movements in the protein would be required for which we have no experimental cues.

DISCUSSION

We have determined the structure of B. subtilis oxalate decarboxylase OxdC in an alternative conformation to that published previously (23). Both structures clearly show the presence of two manganese ions per subunit (Fig. 4). This contrasts with previous evidence from metal analyses using inductively coupled plasma emission spectroscopy that gave values closer to one per subunit (12). Only the open structure has formate bound to site 1 (23), but it is not clear why this was present at all because formate was not specifically added. The key difference of functional significance between the open and closed structures is the alternative conformation of the lid, comprised of amino acids 161–165, at the entrance to the manganese binding site 1. A structural alignment of domain 1 with domain 2 and the plant oxalate oxidase subunit reveals that the existence of the flexible lid near the decarboxylase site 1 entrance is possible due to the insertion of 2 residues (residues 162 and 163).

Identity of the Catalytic Site—It has been suggested by Anand et al. (23) that only the manganese binding site 2 is catalytically active and that Glu-333 is the catalytic proton donor. However, there are a number of problems associated with this hypothesis. On examination of the open structure, it is not clear on what basis Anand et al. (23) state that there is a channel leading to site 2. The water molecules bound to site 2 are not solvent-exposed in either structure, so it is not obvi-
ous how substrates and products could gain access. There is no obvious lid, equivalent to that found in site 1, and the temperature factors around site 2 do not indicate how this site could become solvent-exposed. (It must be noted, however, that both structures were determined at pH 8.0–8.5 and the enzyme is not active above pH 7.5.) Site 1, on the other hand, has a product of the reaction, formate, bound to the manganese ion along with a water molecule. Furthermore the site 1 has access to solvent through a water-filled channel (Fig. 2A). Anand et al. (23) have argued that site 1 could not be catalytically active.

Fig. 4. Comparison of the manganese ion binding sites of OxdC in the two structures and a model. The manganese binding sites of the closed structure (A = site 1 and B = site 2) are shown next to those of the open structure (C = site 1 and D = site 2) (23). A molecular model (E) of oxalate and dioxygen bound to site 1 manganese ion shows the need for Glu-162 to be displaced somewhat in comparison with the experimentally determined closed structure (A). Interatomic distances are shown in Å with dashed lines.
because the open structure did not have an acidic group near the manganese ion that could be the crucial proton donor in the decarboxylase reaction (Fig. 2A). However, the proximity of Glu-162 to the site 1 manganese ion in the closed structure clearly makes this a good candidate for the proton donor (Fig. 2B).

Site-directed mutagenesis has shown that substitution of Glu-162 by Gln or Ala leads to a lowering of both the $V_{\text{max}}$ and $k_{\text{cat}}/K_m$ to 1% that of the wild type enzyme or no detectable activity, respectively (Table II). Such a dramatic effect on the efficiency of the reaction would be expected if Glu-162 were the proton donor of the decarboxylase catalytic cycle and only site 1 were catalytically active. By contrast, the $V_{\text{max}}$ and $k_{\text{cat}}/K_m$ values for the E333A mutant were 6 and 25% that of the wild type enzyme, respectively. This would not be consistent with Glu-333 being the proton donor. This result suggests that although site 2 is not a catalytic site, its disruption does affect catalysis at site 1. The loss of charge coupled with a side chain of comparable size in the E333Q mutant seems to be so disruptive that site 1 is no longer active.

If site 1 were catalytically active, Arg-92 could be expected to be involved in substrate recognition and/or the stabilization of catalytic intermediates. Its substitution by Ala giving an inactive enzyme is consistent with this hypothesis. On the other hand, one would predict that a Lys residue at this position would give a less active, but not inactive, enzyme. This was indeed observed since the $k_{\text{cat}}/K_m$ of the R92K mutant was 7% that of the wild type enzyme. By contrast, substitution of the Arg-270 residue of a catalytically inactive site 2 would be expected to be less detrimental to activity, having only indirect effects on site 1. This was observed with the relative $k_{\text{cat}}/K_m$ values being 3 and 43% for R270A and R270K, respectively. Again the substitution of an Arg by a Lys is the least disruptive. Consistent with this hypothesis, Anand et al. (23) have previously shown that an R270E mutant has 20-fold less activity according to the rate of carbon dioxide production.

It is interesting to note that the oxalate $K_m$ values for all of the active mutants were significantly lower than for the wild type enzyme except for the E162Q mutant. It is perhaps surprising that the values for the mutants, especially the Arg-92 mutants, were not higher given that one would predict that a disrupted active site 1 would bind the substrate less favorably. Nevertheless the fact that the E162Q mutant is the only one with an essentially unaltered $K_m$ for oxalate might be expected given that it is the only active enzyme with a mutation in the lid rather than the main body of the protein. This suggests that the lid and Glu-162 are involved in catalysis but not substrate recognition.

If site 2 is not catalytically active, the question then arises as to why mutations at this site have a significant effect on activity. It should be noted that the minimum distance of 21 Å between any two manganese ions in the structures appears to preclude any electron transfer between them during catalysis. Therefore, the absence of any opportunity for the sites to cooperate catalytically means that the disruption of one by another must have a structural basis. One can envisage this occurring through an altered conformation of the hydrophobic interfaces between the two domains of each subunit. For example, a change in the position of the residue at position 333 could affect the hydrophobic interactions of Leu-332 and Ile-334 with Trp-102 of the neighboring domain. This could in turn affect the conformation of Glu-101, one of the ligands to the site 1 manganese ion. Perhaps the most intimate contact between sites 1 and 2 is between adjacent subunits within each of the two trimers that make up the hexamer. The Trp-96 and Trp-274 residues are both stranded in sequence by 2 of the His residues that bind to the manganese ion in each site (His-95, His-97, His-273, and His-275). The side chains of these Trp residues form stacking interactions with substantial overlap. It is most probably through this interaction that structural changes within one site are transmitted to the other. It is important to note that this type of argument must be invoked whether only site 1, only site 2, or both sites were active given the mutagenesis results. Crystal structures of the mutants will help clarify this issue.

If Glu-162 were the crucial proton donor for the decarboxylase reaction and it is this feature that distinguishes between the oxalate decarboxylase and oxidase enzymes, its substitution might be expected to lead to the conversion of the decarboxylase into an oxidase. In terms of absolute specific activities this was not the case, showing that other changes are necessary to effect a full conversion to give values comparable with that of the plant oxalate oxidase (13 units mg$^{-1}$ (14)). However, it is important to note that only the E162Q mutant retained full oxidase activity, which was 25% that of its own decarboxylase activity. Therefore its oxidase/decarboxylase activity ratio was 2 orders of magnitude higher than for the wild type enzyme. Even more dramatic is the retention of two-thirds of the oxidase activity with the E162A mutant despite it having no decarboxylase activity. To convert the decarboxylase into a fully active oxalate oxidase, additional changes required might include the substitution of Arg-92 by an Asn, the structurally equivalent amino acid in the oxalate oxidase (22).

The only other mutant with detectable oxidase activity was E333A. This result is consistent with the observation by Anand et al. (23) that the activity of this mutant was depressed more strongly in formate production than in carbon dioxide production (25- and 4-fold, respectively). Why this was the case is not clear if only site 1 were catalytically active, but it was the mutant with the highest $V_{\text{max}}$ for the decarboxylase reaction. Nevertheless this result means that the possibility that site 2

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**Table II**

| OxdC (site 1 or 2 mutant) | Oxalate decarboxylase | Oxalate oxidase | Dye oxidation |
|--------------------------|-----------------------|----------------|---------------|
|                          | $V_{\text{max}}$ (units mg$^{-1}$) | $K_m$ (mM) | $k_{\text{cat}}/K_m$ ($\mu$M$^{-1}$ s$^{-1}$) | Specific activity |
| Wild type                | 21.0                   | 16.4          | 952           | 0.06          |
| 1 R92A                   | 0.20                   | 2             | 68            | 0.04          |
| 1 R92K                   | 0.26                   | 8             | 24            | 0.60          |
| 1 E162A                  | 0.24                   | 14            | 12            | 0.06          |
| 1 R92K                   | 0.54                   | 1             | 410           | 0.03          |
| 2 E162Q                  | 1.29                   | 4             | 234           | 0.03          |
| 2 R270A                  | 0.26                   | 8             | 24            | 0.06          |
| 2 R270K                  | 0.24                   | 14            | 12            | 0.06          |
| 2 E333A                  | 0.26                   | 8             | 24            | 0.06          |
| 2 E333Q                  | 0.24                   | 14            | 12            | 0.06          |

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a The limit of detection with the assay conditions used was 0.03 units mg$^{-1}$.
b not determined.
has some oxalate decarboxylase activity as well as site 1 cannot yet be completely ruled out. However, if both were active, the evidence so far strongly suggests that site 1 is the dominant active site.

The dye oxidation activities of all the mutants, except those of Glu-162, are lower than for the wild type enzyme. The substitution of Glu-162 would be expected to disrupt the completion of the normal decarboxylase catalytic cycle. This would allow the accumulation of electron-deficient intermediates that would be capable of the oxidation of dyes. This might also be enhanced if the lid were not able to close properly and isolate the active site. The fact that the E162Q mutant had the highest dye oxidation activity of all (also higher than for the wild type enzyme) is consistent with this possibility. The details of this side reaction are not clear other than it is oxalate-dependent and does not seem to produce hydrogen peroxide (12). It is also not clear why mutants that have lost both oxalate decarboxylase and oxidase activities retain some dye oxidation activity, such as the R92A and E333Q mutants.

Anand et al. (23) have shown that the rate of carbon dioxide production was lowered 13-fold by a Y340F mutation in site 2. It was suggested that this residue could be involved in electron or proton transfer to a peroxy intermediate. However this site 2 residue is not completely conserved in the sequences of confirmed oxalate decarboxylases; the fungal enzymes have Phe at this position (12, 31, 32). The lowering of activity of this mutant would appear to have a structural basis as discussed above.

**Catalytic Cycle**—A working hypothesis for the molecular events during the catalytic cycle of the enzyme at site 1 (Fig. 1) can be drawn up based on the evidence that we have available so far. It would appear that oxalate followed by dioxygen bind to the manganese ion when the lid is open. The binding of oxalate to the site occupied by formate in the open structure could be stabilized by hydrogen bonding interactions with Arg-92 and Tyr-200. There is evidence that the formation of an oxalate radical is concomitant with its deprotonation (18). It is likely that the lid closes most of the way prior to this step to allow proton transfer to Glu-162. The negative charge of the proximal zwitterionic carboxylate radical of the oxalate radical intermediate could be stabilized by Arg-92. The loss of CO₂ would allow Glu-162 to be suitably placed to deliver a proton to the carbon atom of the highly basic anionic formyl radical intermediate.

The closed conformation would allow the isolation of the active site from bulk solvent during catalysis. One purpose of this may be to protect the electron-deficient free radical intermediates of the catalytic cycle from contact with potential reductants. If this were the case, it clearly does not work with complete efficiency because the decarboxylase is capable of the oxalate-dependent oxidation of dyes albeit in only 1 in 350 turnovers. There are examples of other enzymes with reductive active intermediates that possess a lid to prevent access of solvent during catalysis, such as lactate dehydrogenase (33).

The evidence so far cannot completely rule out that site 2 has some catalytic activity. However, it would appear more probable that site 2 has a purely structural role. Site 1 is therefore either the dominant active site or, more likely, the only catalytically active site of OxdC.

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A Closed Conformation of *Bacillus subtilis* Oxalate Decarboxylase OxdC Provides Evidence for the True Identity of the Active Site

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