Kinetic and Spectroscopic Studies on the Quercetin 2,3-Dioxygenase from *Bacillus subtilis*†

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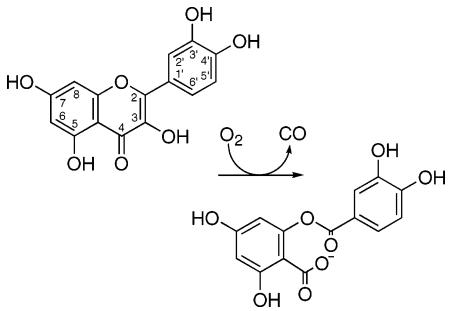
ABSTRACT: Quercetin 2,3-dioxygenase from *Bacillus subtilis* (QueD) converts the flavonol quercetin and molecular oxygen to 2-protocatechuoylphloroglucinolcarboxylic acid and carbon monoxide. QueD, the only known quercetin 2,3-dioxygenase from a prokaryotic organism, has been described as an Fe^{2+}-dependent bicupin dioxygenase. Metal-substituted QueDs were generated by expressing the enzyme in *Escherichia coli* grown on minimal media in the presence of a number of divalent metals. The addition of Mn^{2+}, Co^{2+}, and Cu^{2+} generated active enzymes, but the addition of Zn^{2+}, Fe^{2+}, and Cd^{2+} did not increase quercetinase activity to any significant level over a control in which no divalent ions were added to the media. The Mn^{2+}- and Co^{2+}-containing QueDs were purified, characterized by metal analysis and EPR spectroscopy, and studied by steady-state kinetics. Mn^{2+} was found to be incorporated nearly stoichiometrically to the two cupin motifs. The hyperfine coupling constant of the $g = 2$ signal in the EPR spectra of the Mn^{2+}-containing enzyme showed that the two Mn^{2+} ions are ligated in an octahedral coordination. The turnover number of this enzyme was found to be in the order of 25 s^{-1}, nearly 40-fold higher than that of the Fe^{2+}-containing enzyme and similar in magnitude to that of the Cu^{2+}-containing quercertin 2,3-dioxygenase from *Aspergillus japonicus*. In addition, kinetic and spectroscopic data suggest that the catalytic mechanism of QueD is different from that of the *Aspergillus* quercetinases but similar to that proposed for the extradiol catechol dioxygenases. This study provides evidence that Mn^{2+} might be the preferred cofactor for this enzyme and identifies QueD as a new member of the manganese dioxygenase family.

The flavonol quercetin, isolated from numerous plants, is used in traditional medicine for its antioxidant and antimicrobial properties (*1*). Various strains of the filamentous fungus *Aspergillus* can utilize quercetin and its 3-*O*-glycoside (rutin) as their only carbon source via an extracellular enzyme system (*2*, *3*). The first enzyme in the metabolism of quercetin by this organism is quercetin 2,3-dioxygenase or quercetinase, which catalyzes the oxidative decomposition of quercetin to 2-protocatechuoylphloroglucinolcarboxylic acid and carbon monoxide, as shown in Scheme 1. The quercetinases from *Aspergillus flavus* (*4*), *Aspergillus niger* (*5*), and *Aspergillus japonicus* (*6*) have been characterized, and the crystal structure of the *A. japonicus* quercetinase has been reported (*6*). Recently, the first example of a bacterial quercetinase was reported (*7*, *8*), corresponding to the YxaG protein of *Bacillus subtilis*.

While the sequences of the quercetinases from *A. japonicus* (*6*) and *B. subtilis* share only a 19% sequence identity and 39% similarity, their three-dimensional structures are highly similar (*9*). Both enzymes belong to the cupin superfamily, a family of proteins characterized by their β-barrel tertiary structure (*10*). The cupin domain comprises two conserved motifs with the following characteristic conserved sequences: $G(X)_5HXH(X)_{3,4}E(X)_6G$ and $G(X)_5PXG(X)_2H(X)_3N$, separated by a variable loop of ~20 amino acids (*10*). These two motifs have been found to ligate a number of divalent metal ions (e.g., Mn^{2+}, Cu^{2+}, and Fe^{2+}), which are ligated by two histidines and glutamic acid from motif 1 and a histidine residue from motif 2 (*11*). While the *Aspergillus* quercetinases are Cu^{2+}-containing enzymes, the quercetinase from *B. subtilis* (QueD)[1] has been reported to contain two atoms of Fe^{2+} per subunit, occupying the two cupin-binding domains present in this protein (*9*). Glu 69, His 62, His 624, and His 103 coordinate the first Fe^{2+} ion at the N-terminal cupin motif, and Glu 241, His 234, His 236, and His 275 coordinate the second Fe^{2+} ion at the C-terminal

Scheme 1: Reaction Catalyzed by Quercetin 2,3-Dioxygenase



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cupin motif (*9*). The coordination of Fe^{2+} in QueD is unlike that observed for other Fe^{2+}-dependent cupin dioxygenases, which are usually coordinated by two histidines and a glutamic acid (*12*). The turnover number for this QueD is approximately 2 orders of magnitude lower than that of the quercetinase from *A. flavus* (*13*), which suggests that Fe^{2+} might not be the correct cofactor for this enzyme. Unfortunately, QueD has yet to be isolated from *B. subtilis*, so the natural cofactor is currently unknown. In previous reports, purified QueD overexpressed in *Escherichia coli* has been shown to contain less than 2 equiv of Fe/subunit, and the presence of small amounts of various transition metals was also detected in protein samples (*7*, *8*). In addition, reconstitution of the apoenzyme in the presence of Cu^{2+}, Mn^{2+}, Ni^{2+}, and Co^{2+} resulted in reactivation of the enzyme (*9*). In this report, we describe the expression, purification, and characterization of Mn- and Co-containing QueDs, all with higher activities than that of the Fe^{2+}-containing QueD. We present evidence that Mn^{2+} might be the correct cofactor for QueD and provide insights into the mechanism of this enzyme, in particular, the mechanism of oxygen activation.

MATERIALS AND METHODS

All chemicals were of the highest grade available and, unless otherwise stated, were purchased from Sigma-Aldrich. All electrophoresis equipment and chemicals were purchased from Bio-Rad. Protein concentration was determined using the Bio-Rad DC assay using bovine serum albumin as a standard.

Metal Dependence on the Activity of QueD. One liter cultures of M9 media were inoculated with a 1 mL overnight starter culture (5 mL) of *E. coli* BL21(DE3) pQUER4 (*8*). Kanamycin was added to a final concentration of 30 μg/mL. The cultures were grown at 37 °C and 200 rpm for 6 h, induced with isopropyl β-D-thiogalactopyranoside (IPTG) to a final concentration of 50 mg/L in the presence of 10 μM $ZnSO_4$, $CuCl_2$, $CoCl_2$, $MnSO_4$, $FeCl_2$, $CdCl_2$, or $NiCl_2$, and allowed to grow for an additional 4 h at room temperature (~25 °C). The cells were harvested by centrifugation at 10000*g* for 8 min. The cell paste was resuspended in 10 mL of 50 mM Tris·HCl, pH 7.5, frozen at −80 °C, and thawed. The cells were ruptured using a French press at 7000 psi, and the cell debris was removed by centrifugation at 20000*g* for 10 min. The supernatant was tested for quercetinase activity using the standard assay (*8*), as described below. SDS−PAGE was used to verify that the expression level of protein in each of the cultures was similar and that any difference in activity was not a result of a change in protein concentration.

Purification of Mn- and Co-QueD. E. coli BL21(DE3) pQUER4 was grown in 1 L cultures of M9 minimal media at 37 °C and 200 rpm. Once the cultures reached an OD_{600} of 0.6, the cells were induced with IPTG to a final concentration of 50 mg/L and brought to a final concentration of 1 μM $MnSO_4$ or $CoCl_2$. The cultures were grown for an additional 4 h at 15 °C and harvested by centrifugation at 10000*g* for 5 min. The cell paste was resuspended in 50 mM Tris·HCl, pH 7.5, and stored at −80 °C.

Cells were thawed and ruptured by a French press at 7000 psi in the presence of DNase (1 mg) and phenylmethanesulfonyl fluoride (1 mg). The cell debris was removed by centrifugation at 20000*g* for 15 min. The supernatant was loaded onto a DEAE-Sephacel column (1 × 16 cm), equilibrated with 50 mM Tris·HCl, pH 7.5, and eluted with a NaCl gradient (0−600 mM) in the same buffer. Fractions were collected and assayed for quercetinase activity using the standard assay. The most active fractions were pooled and brought to a 55% saturation level of ammonium sulfate. The suspension was separated by centrifugation at 20000*g* for 20 min. The supernatant was decanted, and the pellet was resuspended in 50 mM Tris·HCl, pH 7.5, and 100 mM NaCl. The protein solution was loaded onto an Ultrogel AcA 34 column (2.5 × 120 cm) and eluted with 50 mM Tris·HCl, pH 7.5, and 100 mM NaCl. The most active fractions were analyzed for purity by SDS−PAGE, pooled, loaded onto a DEAE-Sepharose column (1 × 16 cm) equilibrated with 50 mM Tris·HCl, pH 7.5, and eluted with a gradient of NaCl (100−500 mM) in the same buffer. The most active fractions were analyzed for purity by SDS−PAGE, and the most pure fractions were pooled and concentrated by ultrafiltration using an Amicon Ultra-15 centrifugal filter unit (10000 MWCO). The purified protein was frozen in liquid nitrogen and stored at −20 °C.

Enzyme Assays. The standard activity assay was conducted in 1 mL of 50 mM Tris·HCl, pH 7.5, 100 mM NaCl, 50 μM quercetin, and 5% (v/v) DMSO, and the reaction was initiated by the addition of enzyme. The reaction was monitored by the loss of absorbance of the substrate quercetin at 380 nm (ϵ_{380} = 18500 M^{-1} cm^{-1}) on a HP 8453 photodiode array spectrophotometer. One unit of enzyme activity is defined as the amount of enzyme required to convert 1 μmol of quercetin to product in 1 min at 25 °C.

Initial velocities were measured at varied quercetin and oxygen concentrations by the rate of oxygen consumption using a Clark-type oxygen electrode. The decrease in oxygen concentration was measured with a YSI Model 5300 biological monitor. The temperature of the chamber was maintained at 25 ± 0.1 °C with a circulating water bath. Reaction mixtures (1 mL) contained 50 mM Tris·HCl, pH 7.5, 100 mM NaCl, and 5% (v/v) DMSO at 25 °C and various amounts of quercetin (6−130 μM). The oxygen concentration was varied from 70 to 670 μM by stirring the reaction solution for at least 5 min with premixed O_2/N_2 mixtures in the appropriate proportions to yield the desired O_2 concentration. The resulting oxygen concentration was determined from the known concentration of dissolved oxygen in air-saturated water (258 μM at 25 °C). The reaction was initiated by the addition of 5 μL of enzyme solution varying in concentration from 0.5 to 2 mg/mL. Data from the initial velocity experiments were fit to the sequential rate equation (eq 1) using the programs of Cleland (*14*):

$$v = \frac{V_{max}[A][B]}{K_{iA}K_B + K_A[B] + K_B[A] + [A][B]} \quad (1)$$

[1] Abbreviations: QueD, quercetin 2,3-dioxygenase from *Bacillus subtilis*; Co-QueD, Co-containing quercetin 2,3-dioxygenase; Mn-QueD, Mn-containing quercetin 2,3-dioxygenase; Fe-QueD, Fe-containing quercetin 2,3-dioxygenase; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; EPR, electron paramagnetic resonance; ICP-MS, inductively coupled plasma mass spectrometry; IPTG, isopropyl β-D-thiogalactopyranoside; OxdC, oxalate decarboxylase from *B. subtilis*; ARD, acireductone dioxygenase from *Klebseilla pneumoniae*; MhpB, 2,3-dihydroxyphenylpropionate 1,2-dioxygenase from *Escherichia coli*.

where v is the initial rate of reaction, V_{max} is the maximum rate of reaction at infinite concentration of substrates, K_A and K_B are the Michaelis−Menten constants for the substrates, and K_{iA} is the dissociation constant for the EA complex.

pH Dependence of QueD. The pH profiles for QueD were determined spectrophotometrically on a HP 8453 photodiode array spectrophotometer using a universal buffer system (*15*) consisting of 25 mM tricine, 25 mM MOPS, 25 mM boric acid, and 25 mM citric acid at varying pH and quercetin concentration in air-saturated solutions ([O_2] ~260 μM) at 25 °C. The initial reaction rates were fit to the Michaelis−Menten equation (eq 2) using Kaleidagraph to determine the

$$v = \frac{V_{max}[S]}{K_M + [S]} \quad (2)$$

kinetic parameters V_{max} and K_M over the pH range of 4.5−9. The log(V_{max}) and log(V_{max}/K_M) were plotted versus pH, and the data were fit to eqs 3 and 4 using the program Kaleidagraph (*16*).

$$\log\left(\frac{V_{max}}{K_M}\right)_{app} = \log\left(\frac{V_{max}}{K_M}\right) - \log\left(1 + \frac{[H^+]}{K_{a1}} + \frac{K_{a2}}{[H^+]}\right) \quad (3)$$

$$\log(V_{max})_{app} = \log(V_{max}) + \log\left(1 + \frac{K_a}{[H^+]}\right) \quad (4)$$

Metal Analysis. Determination and quantification of metal content were performed by atomic absorption on a Varian SpectrAA-400 zeeman graphite furnace atomic absorption spectrometer at the Goldwater Environmental Laboratory or by inductively coupled plasma mass spectrometry (ICP-MS) on a Finnigan Element2 ICP-MS. Protein samples were passed through a Chelex 100 column prior to analysis in order to remove any unbound metal ions present in the sample. Protein samples were diluted with deionized water to fall within the standard curve of 0−30 ppb. A sample of buffer alone, diluted in the same manner as the protein sample, was also analyzed.

Inactivation of QueD. A number of metal chelators and inhibitors were tested to determine their effect on the activity of the various forms of QueD. Protein samples were incubated in 50 mM Tris·HCl, pH 7.5, and 100 mM NaCl for 15 min in the presence of 1 mM H_2O_2, NaCN, DTT, EDTA, kojic acid, NaN_3, *O*-ethylxanthic acid, sodium ascorbate, or sodium diethyldithiocarbamate and assayed for residual activity. Enzyme activity was determined using the standard assay, and the reaction was initiated with the addition of 2 μg of enzyme. The resulting reaction rates were compared to a reference of enzyme incubated with buffer in the absence of inhibitor.

EPR Sample Preparation and Analysis. Samples of Mn-QueD (10 mg/mL) and Co-QueD (10 mg/mL) in 50 mM Tris·HCl, pH 7.5, were analyzed by EPR spectroscopy. Three samples of each protein sample were prepared: (1) an anaerobic sample of protein degassed under argon, (2) an anaerobic sample of protein mixed anaerobically with 1 mM quercetin, and (3) an anaerobic sample of protein mixed anaerobically with 1 mM quercetin and exposed to air. Samples were frozen in a hexane/liquid nitrogen slurry in 4 mm standardized quartz EPR tubes. Spectra of metal solutions in buffer were also measured for comparison with the protein spectra.

X-band EPR spectra were recorded on a Bruker ESP-300 E spectrometer with an ER 4116 dual-mode X-band cavity equipped with an Oxford Instruments ESR-900 helium flow cryostat. Spectra were obtained at a microwave frequency of 9.65 GHz. Precise values of the frequency were recorded for each spectra to determine precise g alignment. Spectra were obtained as the sum of five scans at 8 K with a power setting of 2.0 mW and a modulation frequency of 1.26 mT. Subsequent data manipulation was done using IGOR Pro (WaveMetrics, Lake Osewego, OR).

RESULTS

Protein Expression and Purification. Quercetin 2,3-dioxygenase from *B. subtilis* overexpressed in *E. coli* has been identified as an Fe^{2+}-containing dioxygenase (*7*, *8*). However, as QueD has not yet been purified from *B. subtilis*, the identity of the metal cofactor in the native enzyme is currently unknown. As the incorporation of the correct metal at the active site of overexpressed cupin enzymes in *E. coli* has been shown to require the addition of the particular metal ion to the culture media at the time of induction (*17*, *18*), the effect of a number of divalent metals in the quercetinase activity of QueD when overexpressed in *E. coli* was determined. Cell cultures of *E. coli* expressing QueD were grown in minimal media, and a number of divalent metal salts were added at the time of induction. It was found that the addition of Mn^{2+}, Co^{2+}, Ni^{2+}, and Cu^{2+} salts resulted in a 35-, 24-, 2.6-, and 1.4-fold increase, respectively, in quercetinase activity in crude cell extracts over a control in which no metal was added. The addition of Zn^{2+}, Fe^{2+}, and Cd^{2+} did not increase quercetinase activity in crude extracts to any significant level over the control.

Samples of QueD generated in the presence of Mn^{2+} and Co^{2+} were purified for metal analysis and kinetic and spectroscopic characterization. A sample of QueD purified from LB media, as described by Barney et al. (*8*), was used as a control. The optimized expression of soluble and active enzymes required lowering the temperature of the cultures at the time of induction to 15 °C to prevent the formation of inclusion bodies. In each enzyme preparation, the amount of purified protein obtained from 10 g of cell paste was between 30 and 40 mg. Atomic absorption and ICP-MS analysis revealed that the protein induced in the presence of Mn^{2+} contained 1.6−1.9 atoms of Mn/subunit, with trace amounts of iron and other transition metals. QueD induced in the presence of Co^{2+} only contained between 0.65 and 0.80 atom of cobalt and 0.1 atom of iron per subunit. Metal reconstitution experiments to enhance the occupancy of Co^{2+} in the protein samples have been unsuccessful.

Inactivation of QueD. To confirm that the activity of Mn-QueD and Co-QueD was the result of the respective incorporation of Mn^{2+} and Co^{2+}at the active site, the enzymes were incubated with various metal chelators and inhibitors, and the effect of these compounds on enzyme activity was determined (Table 1). Mn-QueD was not significantly affected by any of the compounds tested. *O*-ethylxanthanate, which has been shown to ligate cobalt and copper (*4*, *19*), significantly inactivated the Co-QueD. Barney et al. (*8*) have

Table 1: Enzyme Inactivation[a]

	metal-containing QueD	
	Co^{2+}	Mn^{2+}
kojic acid	92	100
H_2O_2	93	82
NaCN	91	89
O-ethylxanthic acid	69	100
diethyldithiocarbamate	90	84
EDTA	110	90
DTT	110	96
sodium ascorbate	110	80
NaN_3	110	100

[a] Percent of activity remaining after 15 min of incubation with 1 mM reagent. The reported percentage is the average of three measurements.

Table 2: Kinetic Parameters of Quercetin 2,3-Dioxygenase

metal[a]	atoms/subunit	k_{cat} (s^{-1})	K_M (μM)	$K_M(O_2)$ (μM)
Mn	1.8	25 ± 1	4.0 ± 0.9	90 ± 10
Co	0.65	6.7 ± 0.2	7.5 ± 0.5	79 ± 5
Fe	0.80[b]	0.65 ± 0.02	5.2 ± 0.6	150 ± 9

[a] The metal occupying the active sites of quercetin 2,3-dioxygenase. [b] As reported by Barney et al. (*8*).

reported that incubation of Fe-QueD with 1.5 mM *O*-ethylxanthanate resulted in a 15% inhibition. The copper-specific inhibitor diethyldithiocarbamate (*4*), did not affect the activity of either the Co-QueD or Mn-QueD, as has been shown for the Fe-QueD (*8*). Hydrogen peroxide, which has been shown to significantly inhibit Fe^{2+}-dependent enzymes (*20*), had little effect on the activity of the Co-QueD or Mn-QueD. Also, EDTA did not inhibit any of the two forms of QueD (Mn or Co) or the Fe-QueD, as has been previously shown (*8*). The combined results from these inhibition studies strongly suggest that catalysis by Mn-QueD and Co-QueD is dependent on Mn^{2+} and Co^{2+}, respectively.

Steady-State Kinetics. The kinetic parameters for Mn-QueD, Co-QueD, and Fe-QueD are presented in Table 2. These kinetic parameters were determined by varying the concentration of both substrates (quercetin and oxygen), unlike the apparent kinetic parameters previously reported for Fe-QueD, which were determined at a single concentration of O_2 (*8*, *9*, *21*). The k_{cat} for the three enzymes vary from 25 s^{-1} for the Mn-QueD to 0.7 s^{-1} for the Fe-QueD. The K_M for quercetin, at around 4 μM, is nearly identical among all QueDs. The K_M for oxygen for Mn-QueD and Co-QueD was also found to be nearly identical ($\sim$80 μM), while it was slightly higher for the Fe-QueD ($\sim$140 μM).

pH Dependence of QueD. The pH dependence for V_{max}/K_M and V_{max} was determined by measuring the initial rates at varying concentrations of quercetin at 25 °C and air-saturated buffer ($[O_2] \approx 280$ μM). At pHs above 8.5, the uncatalyzed decomposition reaction of quercetin is significant, preventing the accurate determination of the kinetic parameters. It is noted that the autoxidation of quercetin in alkaline solution yields a number of products including 2,4,6-trihydroxyphenylglycolic acid, protocatechuic acid, phloroglucinol, and 2,4,6-trihydroxybenzoic acid (*22*). As shown in Figure 1, the V_{max} pH profile drops at low pH but plateaus at high pH. The pK_a determined from the V_{max} profile is 5.6 $\pm$ 0.1, indicating that a single ionizable group participates in catalysis. The V_{max}/K_M pH profile is bell shaped, with a

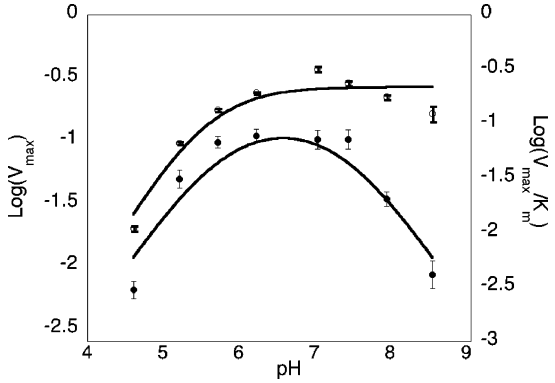


FIGURE 1: pH dependence on kinetic parameters V_{max}/K_M (●) and V_{max} (○) for the Mn^{2+}-containing quercetin 2,3-dioxygenase. The curves were generated by a fit to eqs 3 and 4, respectively.

low pK_a of 5.8 $\pm$ 0.1, nearly identical to the pK_a found in the V_{max} pH profile, and a high pK_a of 7.3 $\pm$ 0.1, consistent with the pK_a of the 4′-hydroxy group of quercetin (*23*, *24*). This suggests that the 4′-hydroxide group must be protonated for quercetin to bind to QueD. The V_{max} and V_{max}/K_M pH profiles of Co-QueD were superimposable to those of the Mn-QueD.

EPR Spectral Analysis. The X-band EPR spectrum of Mn-QueD at 8 K is shown in Figure 2A. The spectrum contains a strong 6-fold hyperfine splitting centered at $g = 2$, corresponding to the $+^1/_2 \rightarrow -^1/_2$ transition of the $^5/_2$ spin of ^{55}Mn. The hyperfine coupling constant measured directly from the spacing of the most intense derivative trough is approximately 93 G. This hyperfine coupling constant corresponds well to an octahedrally coordinated Mn^{2+} with nitrogen or oxygen ligands (*25*). In addition to the $g = 2$ signal, there is also a less intense 6-fold hyperfine signal centered at $g = 9$ (Figure 2B). The signal at $g = 9$ is consistent with two overlapping six-line signals that arise from two isolated Mn^{2+} ions (*26*). The hyperfine coupling constant of 92 G for the $g = 9$ signal is nearly identical to that of the $g = 2$ signal. No other signals were present in the spectra. Attempts to simulate the features in the spectrum have been unsuccessful. The spectrum reported by Bowater et al. (*7*) for the Fe^{2+}-containing QueD clearly shows a similar signal for Mn^{2+}, even though this sample is reported to contain less than 0.05 atom of Mn. The addition of quercetin under anaerobic conditions resulted in only slight changes in the coupling at both the low- and high-field signals, suggesting that no change in the oxidation state of Mn^{2+} occurs upon substrate binding.

The X-band EPR spectrum of Co-QueD at 8 K is shown in Figure 2C. ^{59}Co, with its $S = ^7/_2$ spin system, gives rise to an 8-fold hyperfine splitting with $g = 6.5$. There is an adventitious iron signal at $g = 4.3$.

DISCUSSION

Recombinant quercetin 2,3-dioxygenase from *B. subtilis* has been characterized as an Fe^{2+}-dependent dioxygenase (*8*, *21*). The recent crystal structure of this enzyme has confirmed the presence of two atoms of Fe^{2+}, each occupying a cupin-binding domain in this bicupin enzyme (*9*). However, the identity of the preferred cofactor for QueD remains unknown, as the native protein from *B. subtilis* has yet to be purified and characterized. Several observations suggest

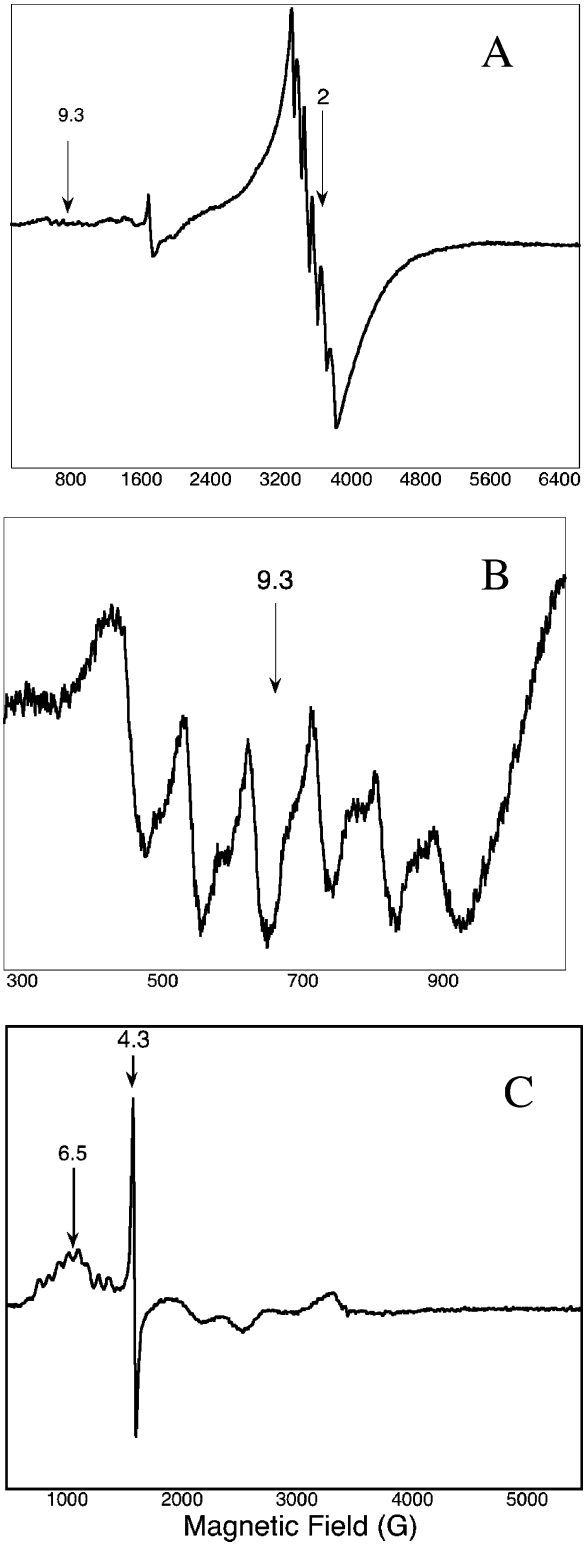


FIGURE 2: EPR spectra of quercetin 2,3-dioxygenase from *B. subtilis*. (A) X-band EPR spectrum of Mn^{2+}-containing quercetin 2,3-dioxygenase at 8 K in 50 mM Tris·HCl, pH 7.5. (B) Enlargement of the high-field ^{55}Mn signal resulting from a spin isolation in the enzyme. (C) X-band EPR spectrum of Co^{2+}-containing quercetin 2,3-dioxygenase at 8 K in 50 mM Tris·HCl, pH 7.5.

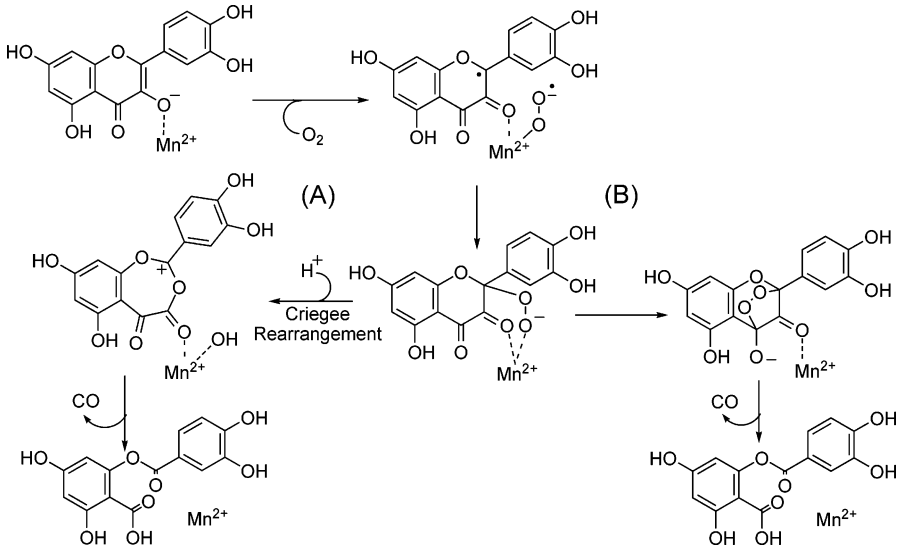
that Fe^{2+} might not be the correct cofactor for this enzyme. First, the k_{cat} of the Fe^{2+}-containing QueD is approximately 100-fold lower than that of the quercetinase from *A. flavus* (*13*). Second, reconstitution of QueD in the presence of Fe^{2+} has failed to reactivate the apoenzyme (*9*). Finally, while the Fe^{2+} ions in QueD are ligated by three histidines and a glutamic acid, crystal structures of other Fe^{2+}-containing cupin enzymes have shown that Fe^{2+} is usually coordinated by only two histidines and a glutamic or aspartic acid (*27*−*35*). The ligand environment in QueD most closely resembles that of cupin enzymes known to ligate Mn^{2+} or Ni^{2+} [e.g., oxalate decarboxylase (OxdC) (*36*) and aci-reductone dioxygenase (ARD) (*37*)].

The expression of recombinant metalloenzymes in *E. coli* is challenging, as the native mechanism of metal transport might not be present in this host bacterium. Once *E. coli* is induced to produce recombinant proteins, the incorporation of metals in the active site is controlled by the concentration of metals in the culture media (*17*, *38*). As the concentration of iron in *E. coli*, when grown on LB broth, is 20−30-fold higher than that of manganese and other transition metal ions (*39*), iron regularly occupies the active site of metalloenzymes overexpressed in this organism. This is best illustrated by the Ni^{2+}-containing aci-reductone dioxygenase from *Klebseilla pneumoniae*, also a member of the cupin superfamily. When overexpressed in *E. coli* grown in LB broth, ARD has been shown to contain Fe^{2+}, which catalyzes a different oxidation reaction on the same substrate as the Ni^{2+}-containing ARD (*40*). The addition of Ni^{2+} at the time of induction is required to produce Ni^{2+}-containing ARD. It is interesting to note that ARD also can function with Co^{2+} and Mg^{2+}, replacing the Ni^{2+} and Fe^{2+}, respectively (*40*).

In the course of this work it was found that the inclusion of four different metal ions (manganese, cobalt, nickel, and copper) in the media resulted in isolated QueDs with the respective metal ions incorporated, and these enzymes displayed higher turnover numbers than that of the Fe^{2+}-containing enzyme. All of these transition metal ions have been previously found to regenerate activity in samples of apo-QueD (*9*). While the K_Ms for quercetin and oxygen for the Mn- and Co-QueD were found to be similar, the k_{cat} for these enzymes were different and significantly higher to that of the Fe-QueD (Table 2). In fact, the k_{cat}/K_M for the Mn-QueD is similar to that found for the quercetinase from *A. flavus* (unpublished data). Inhibition studies with specific metal chelators demonstrated that the activity of the Mn- and Co-QueD was the result of the presence of Mn^{2+} and Co^{2+}, respectively, in the active site of the enzymes.

Insights into the mechanism of the *Aspergillus* quercetinases have been obtained by EPR spectroscopy (*15*) and crystal structure studies (*6*, *41*). In the proposed mechanism for these dioxygenases, the Cu^{2+} center activates quercetin for direct reaction with dioxygen. This is because activation of dioxygen by the metal ion is not possible; the copper ion in its oxidized state is not able to bind oxygen directly. The presence of a metal ion in the reduced state in QueD suggests a different mechanism for this enzyme. A plausible mechanism for oxygen activation by this enzyme, similar to that proposed for the Fe^{2+}- and Mn^{2+}-extradiol catechol dioxygenases (*42*) and the oxalate decarboxylase from *B. subtilis* (*17*, *43*), is one in which both substrates, flavonol and dioxygen, ligate to the metal ion before oxygen activation. The metal ion, in this case, acts like an electron conduit by which an electron is transferred from the reduced metal to oxygen. Electron transfer from quercetin to the oxidized metal center would generate a quercetin radical−M^{2+}−superoxide species (Scheme 2). The steady-state concentration of such species could be so small that the change in

Scheme 2: Mechanism of Quercetin 2,3-Dioxygenase from *B. subtilis*



oxidation state in the metal center would not be detectable by EPR, explaining the lack of change in the EPR signal when an anaerobic enzyme−flavonol complex is exposed to air. It should be noted that the *A* value of 93 found for the $g = 2$ signal in the EPR spectra of Mn-QueD is indicative of octahedral coordination, closely matching the coordination found for Mn^{2+} in OxdC (*36*), even though the Fe^{2+} in the structure of QueD was assigned to have a trigonal bipyramidal coordination (*9*). An octahedral coordination for the Mn^{2+} ions in QueD supports the proposal that both substrates bind to the metal center before oxygen is activated. By replacing the two hypothetical equatorial water ligands, the quercetin radical−M^{2+}−superoxide species would be able to react with minimum movement of the substrates.

After the formation of the quercetin radical−M^{2+}−superoxide intermediate, the reaction could proceed via two pathways (Scheme 2, A and B). In the first pathway (A), a dioxolane intermediate is formed and decomposes into products, similar to the mechanism proposed for the *Aspergillus* quercetinase (*41*). In the second pathway (B), the superoxide intermediate reacts with the quercetin radical to form at lactone intermediate and a hydroxide ion via a Criegee intermediate. A Baeyer−Villiger rearrangement with alkyl migration would then generate the final products. This is identical to the mechanism proposed for extradiol catechol dioxygenases (*42*) and aci-reductone dioxygenase (*18*).

The pH profiles of QueD could differentiate between these two reaction schemes. The formation and decomposition of a dioxolane are theoretically pH independent (Scheme 2, A). This is observed in the pH profile of V_{max} of *A. flavus* (unpublished data). However, the pH profile for V_{max} and V_{max}/K_M for QueD indicates that the catalytic reaction requires one or more groups on the enzyme or substrate to be in a given protonation state. A similar pK_a has been found in the V_{max} pH profile for 2,3-dihydroxyphenylpropionate 1,2-dioxygenase (MhpB) from *E. coli*, a member of the Fe^{2+}-dependent type III extradiol catechol dioxygenases also belonging to the cupin superfamily (*44*). In the V_{max} pH profile of MhpB, the amino acid responsible for the pK_a of 6.4 has been identified as a histidine at position 179, which participates in a proton extraction from the catechol substrate. A proton abstraction from the quercetin is unlikely, but a proton transfer from an amino acid is plausible in the formation of a lactone intermediate. Unfortunately, no histidine residues, besides those involved in metal chelation, reside in close proximity to the metal center in QueD to indicate participation in catalysis (*9*). It is plausible that once substrates bind to the metal center, a conformational change occurs in which a residue, most likely a histidine, moves toward the metal substrate complex in order to participate in catalysis. A similar conformation change has been observed in the structurally similar OxdC, in which the enzyme undergoes a drastic conformation change following binding of oxalate and oxygen allowing a glutamic acid at position 162 to participate in catalysis (*45*, *46*). Further work is necessary to elucidate the group responsible for the pK_a found in both V_{max} and V_{max}/K_M pH profiles of QueD.

We conclude that quercetin 2,3-dioxygenase from *B. subtilis* is a dioxygenase that can function with a number of different divalent metals, although Mn^{2+} appears to be the preferred cofactor for this enzyme. Important differences have been found between QueD and the *Aspergillus* quercetinases, particularly in the mechanism of oxygen activation. In *Aspergillus* quercetinase, like the intradiol catechol dioxygenase (*42*), the substrate is first activated by the metal ion to allow for its direct reaction with dioxygen. In QueD, similar to the extradiol catechol dioxygenase (*47*), the metal ion acts as a conduit for electron transfer from the substrate to oxygen.

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