SUPPORTING INFORMATION

Dissecting the Total Transition State Stabilization Provided by Amino Acid Side-Chains at Orotidine 5'-Monophosphate Decarboxylase: A Two-Part Substrate Approach

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Materials. Orotidine 5'-monophosphate trisodium salt (99%) was purchased from Sigma or was prepared from uridine 5'-monophosphate by Dr. Krisztina Toth using modifications of literature procedures (*1-3*). 1-(β-D-Erythrofuranosyl)orotic acid and 1-(β-D-erythrofuranosyl)uridine were available from an earlier study (*4*). 6-Azauridine 5'-monophosphate (free acid, > 99% by HPLC) was purchased from R. I. Chemical Inc. Sodium phosphite (dibasic, pentahydrate), 3-(N-morpholino)propanesulfonic acid (MOPS, ≥ 99.5%) and ammonium acetate (≥ 99%) were purchased from Fluka. Water was from a Milli-Q Academic purification system. All other chemicals were reagent grade or better and were used without further purification.

Preparation of Solutions. Solution pH was determined at 25 ˚C using an Orion Model 720A pH meter equipped with a Radiometer pHC4006-9 combination electrode that was standardized at pH 7.00 and 10.00 at 25 ˚C.

Stock solutions of orotidine 5'-monophosphate (OMP) and 6-Azauridine 5'-monophosphate (6-aza-UMP) were stored in small aliquots at -20 ˚C. The stock solution of 1-(β-D-erythrofuranosyl)orotic acid (EO, 28 mM) was prepared by dissolution of the free acid and neutralization to pH ≈ 6 by the addition of 1 M NaOH and was stored at -20 ˚C. The concentration of OMP was determined from the absorbance in 0.1 M HCl at 267 nm using ε = 9430 M^{-1} cm^{-1} (*5*). The concentration of EO was determined from the absorbance in 0.1 M HCl at 267 nm using ε = 9570 M^{-1} cm^{-1} reported for orotidine (*5*).

The stock solution of phosphite buffer (100 mM, 80% free base, I = 0.28) was prepared by addition of a measured amount of 1 M HCl to the sodium salt to give the desired acid/base ratio. MOPS buffers were prepared by addition of measured amounts of 1 M HCl and solid NaCl to give the desired acid/base ratio and final ionic strength.

Kinetic Parameters for Decarboxylation of Orotidine 5'-Monophosphate Catalyzed by S154A, Q215A and S154A/Q215A Mutant Yeast OMPDCs. All enzyme assays were conducted under the following conditions: 10 mM MOPS (50% free base) pH 7.1, at 25 ˚C and I = 0.105 (NaCl). The concentration of mutant OMPDC in the stock solution was determined from its absorbance at 280 nm using an extinction of 29,910 M^{-1} cm^{-1} that was calculated using the ProtParam tool available on the ExPASy server (*6*).

Initial velocities, v_o (M s^{-1}), of decarboxylation of OMP (10 - 400 μM) catalyzed by the S154A, Q215A and S154A/Q215A mutants of OMPDC from *Saccharomyces cerevisiae* that also contain the S2H, C155S, A160S and N267D mutations (see below) were determined spectrophotometrically by following the decrease in absorbance at 279 nm (Δε= -2400 M^{-1} cm^{-1}) (*7*) or 290 nm (Δε = -1620 M^{-1} cm^{-1}). Assays in a total volume of 1 mL were initiated by the addition of 1 - 10 μL of a stock solution of mutant OMPDC to give a final enzyme concentration

([E]) of 1.6 μM (S154A), 11 - 23 nM (Q215A) or 3.9 μM (S154A/Q215A). Values of k_{cat} and K_m were obtained from the nonlinear least squares fits of the values of v_o/[E] (s^{-1}) to the Michaelis-Menten equation (Figures S1 - S3). The resulting values of k_{cat} and K_m are given in Table 1 of the main text.

Decarboxylation of 1-(β-D-Erythrofuranosyl)orotic Acid Catalyzed by S154A, Q215A and S154A/Q215A Mutant Yeast OMPDCs Followed by HPLC. Samples of mutant yeast OMPDCs that had been stored at -80 ˚C were defrosted and extensively dialyzed at 4 ˚C against 100 mM MOPS (50% free base) pH 7.1 at I = 0.28 (NaCl).

The decarboxylation of 5 - 14 mM of the truncated substrate 1-(β-D-erythrofuranosyl)orotic acid (EO) in 50 mM MOPS (50% free base) at pH 7.1, 25 ˚C and I = 0.15 (NaCl) catalyzed by S154A, Q215A or S154A/Q215A mutant yeast OMPDC was followed in a discontinuous assay in which the initial velocity of formation of the product 1-(β-D-erythrofuranosyl)uridine (EU) was monitored by HPLC. Reactions in a total volume of 160 μL were initiated by the addition of 80 μL of a stock solution of the enzyme (see above) to 80 μL of a solution of EO in water to give a solution containing 50 mM MOPS, 5 - 14 mM EO and 60 μM (Q215A) or 200 - 260 μM (S154A and S154A/Q215A) mutant OMPDC at pH 7.1 and I = 0.15 (NaCl). The concentration of the mutant OMPDC in the reaction mixture was determined by standard assay (see below), and periodic standard assay showed that there was no significant decrease in the activity of the enzyme during the reaction. The reactions were followed for 2 - 6 hours, during which time there was up to 1% reaction (Q215A) or ca. 0.05% reaction (S154A and S154A/Q215A). At various times an aliquot (20 μL) was withdrawn and the reaction halted by its addition to 100 μL of 0.12 mM 6-azauridine 5'-monosphosphate (6-aza-UMP), a potent competitive inhibitor, to give a 3 - 10-fold excess of 6-aza-UMP over enzyme. The enzyme-

inhibitor complex was removed by ultrafiltration using an Amicon Micron filtration device (10K MWCO). 100 μL of the filtrate was analyzed by HPLC using a Waters Atlantis dC_{18} 3 μm column (3.9 x 150 mm) with an isocratic flow of 1 mL/min 10 mM NH_4OAc (pH 4.2) and peak detection at 262 nm. Under these conditions the unreacted EO elutes close to the void volume and the product EU elutes at 7.5 min. The concentration of EU in the HPLC sample was obtained from the integrated peak area via interpolation of a calibration curve that was constructed using authentic EU.

Initial velocities of decarboxylation of EO, v_o (M s^{-1}), were determined as the slopes of linear plots of the concentration of the product EU in the decarboxylation reaction mixture against time (Figure S4). Second-order rate constants k_{cat}/K_m (M^{-1} s^{-1}) for enzyme-catalyzed decarboxylation of EO were determined as the slopes of plots of v_o/[E] against $[EO]_o$ (Figure S5). The absence of curvature in these plots shows that $[EO]_o << K_m$.

Decarboxylation of 1-(β-D-Erythrofuranosyl)orotic Acid Catalyzed by S154A, Q215A and S154A/Q215A Mutant Yeast OMPDCs in the Presence of Phosphite Dianion Followed by HPLC. Samples of mutant yeast OMPDCs that had been stored at -80 ˚C were defrosted and extensively dialyzed at 4 ˚C against 50 mM MOPS (45% free base) pH 7.0 at I = 0.12 (NaCl).

The decarboxylation of 2 mM EO in the presence of 10 mM MOPS (45% free base, pH 7.0) and various concentrations of phosphite buffer (80% dianion) at pH 7.0, 25 ˚C and I = 0.15 (NaCl) catalyzed by S154A, Q215A or S154A/Q215A mutant yeast OMPDC was followed in a discontinuous assay in which the initial velocity of formation of the product EU was monitored by HPLC. Reaction mixtures in a total volume of 200 or 300 μL were prepared by mixing stock solutions of 50 mM MOPS buffer (45% free base, pH 7.0), 100 mM sodium phosphite (80% free base, pH 7.0, I = 0.28, NaCl), 0.28 M sodium chloride, EO and water to give final concentrations

of 2 mM EO, 10 mM MOPS, and 12.5 - 50 mM phosphite buffer (80% free base) at pH 7.0 and I = 0.15 (NaCl). The reactions were initiated by the addition of 3 - 30 μL of a stock solution of the enzyme (see above) to give a final concentration of 2 μM (Q215A) or 20 μM (S154A and S154A/Q215A) mutant OMPDC. The concentration of the mutant OMPDC in the reaction mixture was determined by standard assay (see below), and periodic standard assay showed that there was no significant decrease in the activity of the enzyme during the reaction. The reactions were followed for 2 - 6 hours, during which time there was up to 10% reaction (Q215A) or ca. 0.5% reaction (S154A and S154A/Q215A). At various times an aliquot (20 μL) was withdrawn and the reaction halted by its addition to 100 μL of 0.12 mM 6-aza-UMP, to give a 30 - 300-fold excess of 6-aza-UMP over the enzyme. The enzyme-inhibitor complex was removed by ultrafiltration and the filtrate was analyzed by HPLC, as described above for the reactions in the absence of phosphite dianion.

Initial velocities of decarboxylation of EO, v_o (M s^{-1}), were determined as the slopes of linear plots of the concentration of the product EU in the decarboxylation reaction mixture against time. Observed second-order rate constants $(k_{cat}/K_m)_{app}$ (M^{-1} s^{-1}) for enzyme-catalyzed decarboxylation of EO at a given concentration of phosphite dianion were calculated as $(k_{cat}/K_m)_{app} = v_o/[E][EO]_o$. Third-order rate constants for enzyme-catalyzed decarboxylation of EO activated by phosphite dianion were determined as the slopes of plots of $(k_{cat}/K_m)_{app}$ (M^{-1} s^{-1}) against the concentration of phosphite dianion (Figure S6).

Standard Enzyme Assays for the Activity of Mutant OMPDCs. The activity of mutant OMPDC in the reaction mixtures for the decarboxylation of EO that were monitored by HPLC was determined from the initial velocity, v_o (M s^{-1}), of decarboxylation of OMP (100 μM) in 10 mM MOPS (50% free base) pH 7.1 at 25 ˚C and I = 0.105 (NaCl), monitored

spectrophotometrically at 279 nm ($\Delta\varepsilon$= -2400 M^{-1} cm^{-1}). Assays in a total volume of 1 mL were initiated by the addition of 4 - 50 μL of the neat reaction mixture or of an appropriate dilution to give a final enzyme concentration of 1 - 2 μM (S154A), 20 - 30 nM (Q215A) or 1 - 4 μM (S154A/Q215A). Values of V_{max} (M s^{-1}) were calculated from the observed values of v_o using the appropriate value of K_m (Table 1). The concentration of mutant OMPDC in the assay mixture was calculated from the value of V_{max} using the relationship $[OMPDC]_{assay} = V_{max}/k_{cat}$, with the appropriate value of k_{cat} (Table 1).

Cloning and Site-Directed Mutagenesis of Yeast Orotidine 5'-Monophosphate Decarboxylases (OMPDCs). The plasmid pODC-C155S containing the gene encoding the C155S mutant of OMPDC from *Saccharomcyes cerevisiae* was a gift from Dr. Steven Short (GlaxoSmithKline). This plasmid was digested with the *Nde*I and *Bam*HI restriction enzymes and the OMPDC gene was subcloned into the pET-15b expression vector (Novagen; encoding an N-terminal His_6-tag) via the *Nde*I and *Bam*HI restrictions sites at the 5'- and 3'-ends, respectively, to produce the plasmid pScODC-15b. The sequence of the gene encoding yeast OMPDC contained in pScODC-15b was verified at the University of Illinois, Urbana-Champaign Core Sequencing Facility. The protein sequence differs from the published sequence for wildtype yeast OMPDC by the following mutations: S2H (*8*), C155S (*9*), A160S and N267D (*8*). Except for the C155S mutation, the sequence is the same as that observed in the published crystal structure of wildtype yeast OMPDC (*10*). The C155S mutation results in a more stable protein but does not affect the kinetic parameters or the overall structure of the enzyme (*9*).

Site-directed mutagenesis on pScODC-15b to introduce the S154A, Q215A and S154A/Q215A mutations was carried out using the QuikChange II kit from Stratagene. The primer used to introduce the S154A mutation was:

5'-GGCCTTTTGATGTTAGCAGAATTGGCATCTAAGGGCTCCC-3'

and the primer used to introduce the Q215A mutation was:

5'-GACAAGGGAGACGCATTGGGTGCACAGTATCGTACCGTGG-3'

No additional primers were necessary for introduction of the S154A/Q215A double mutation. For the S154A and Q215A mutations, 30 ng of the plasmid pScODC-15b containing the gene for C155S mutant yeast OMPDC that had been purified from *dam*$^+$ XL1-Blue cells was added to a PCR reaction mixture containing 5 μL 10x *Pfu* Ultra buffer, 125 ng each of the forward and reverse mutagenesis primers, 1 μL of the 20 mM dNTP mixture, 2.5 units of *Pfu* Ultra HF DNA polymerase and water to give a final volume of 50 μL. The parameters for the PCR were: 30 sec at 95 ˚C followed by 19 cycles of 30 sec at 95 ˚C, 1 min at 55 ˚C and 13 min at 68 ˚C. Following the PCR, 20 units of the *Dpn*I restriction enzyme were added and the reaction mixture was incubated at 37 ˚C for 3 hr in order to degrade the methylated template DNA. One microliter of the reaction mixture was electroporated into XL1-Blue cells and a single colony was used for mutant plasmid purification and sequencing at the UIUC Core Sequencing Facility. The same procedure was used to introduce the S154A/Q215A double mutation except that the plasmid encoding the S154A mutation was used as the template.

The plasmid containing the gene for S154A mutant OMPDC was digested with the *Nde*I and *Bam*HI restriction enzymes and the restriction fragment was ligated into the plasmid pKKHis10, derived from pKK223-3 (Amersham), and which encodes an N-terminal His_{10}-tag, to produce a plasmid coding for the S154A mutant yeast OMPDC with an N-terminal His_{10}-tag.

Expression and Purification of S154A Mutant Yeast OMPDC. The plasmid coding for S154A mutant OMPDC with an N-terminal His_{10}-tag was electroporated into a strain of *Escherichia coli* in which the gene encoding OMPDC was disrupted using the Datsenko and

Wanner method (*11*). The transformed cells were grown in LB medium (4 L) at 20 ˚C for approximately 48 hr. The cells were harvested by centrifugation at 4,600 x g and resuspended in 140 mL of binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9). The cells were lysed by sonication with a 550 Sonic Dismembrator (Fisher Scientific) for 12 min (5 sec pulses separated by 10 sec) and cellular debris was removed by centrifugation for 45 min at 31,000 x g. The cleared supernatant was loaded onto a column of Ni-Chelating Sepharose Fast Flow (Pharmacia Biotech; 150 mL volume) that was equilibrated with the binding buffer. The column was washed with 350 mL of binding buffer followed by 450 mL of binding buffer containing 0.2 M imidazole. The protein was eluted from the column with 400 mL of strip buffer (100 mM EDTA, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9). Fractions containing the His_{10}-tagged S154A mutant OMPDC were identified by SDS-PAGE, pooled, and dialyzed at 5 ˚C against 20 mM Tris-HCl, pH 7.9.

A 10-fold dilution of 10X phosphate buffered saline (PBS, pH 7.4) was made into the protein solution and the N-terminal His_{10}-tag was removed by the action of thrombin (1 unit/mg mutant OMPDC) at room temperature for ca. 16 hr. The thrombin cleavage reaction was checked for completion using SDS-PAGE and the resulting S154A mutant OMPDC was dialyzed at 5 ˚C against the storage buffer (100 mM NaCl, 20% glycerol, 20 mM Tris-HCl, pH 7.9). The protein was concentrated to ca. 25 mg/mL by ultrafiltration, flash frozen in liquid N_2 as ca. 25 μL pellets, and stored at -80 ˚C.

Expression and Purification of Q215A and S154A/Q215A Mutant Yeast OMPDCs. The Q215A and S154A/Q215A mutant OMPDCs did not express well in the strain of *E. coli* in which the gene encoding OMPDC has been disrupted. Therefore these mutant proteins were expressed via transformation of *E. coli* BL21 (DE3) with the plasmids coding for the Q215A and

S154A/Q215A mutants OMPDCs with an N-terminal His_6-tag. The cultures were grown in LB medium (4 L) at 20 ˚C for 48 hr, with the addition of 0.5 mM IPTG after 24 hours of growth to induce the expression of OMPDC. The cells were harvested, sonicated, resuspended in binding buffer and the cellular debris removed, as described above for the S154A mutant. Each supernatant was loaded onto a column of Ni-Sepharose (150 mL) that was equilibrated with the binding buffer. The column was washed with 350 mL of binding buffer followed by 300 mL of wash buffer (60 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9). The protein was eluted from the column using a gradient (400 mL) of 60 mM to 1 M imidazole in the wash buffer. Fractions containing the His_6-tagged mutant OMPDC were identified by SDS-PAGE, pooled, and dialyzed at 5 ˚C against 20 mM Tris-HCl, pH 7.9. 10X PBS was added to the protein solution and the N-terminal His_6-tag was removed by the action of thrombin, as described above for the S154A mutant. The protein was dialyzed at 5 ˚C against 20 mM Tris-HCl, pH 7.9 and loaded onto a Q Sepharose High Performance column (Pharmacia Biotech; 50 mL volume) that was equilibrated with 20 mM Tris-HCl, pH 7.9. The column was washed with 50 mL of 20 mM Tris-HCl, pH 7.9 and the protein was eluted using a gradient (500 mL) of 0 to 0.5 M NaCl in 20 mM Tris-HCl, pH 7.9. Fractions containing the mutant OMPDC were identified by SDS-PAGE, pooled, dialyzed at 5 ˚C against the storage buffer, concentrated and flash frozen, as described above for the S154A mutant.

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FIGURE LEGENDS

Figure S1. Dependence of v_o/[E] (s^{-1}) for the decarboxylation of OMP catalyzed by S154A mutant yeast OMPDC (1.6 μM) on the concentration of OMP in 10 mM MOPS at pH 7.1, 25 ˚C and I = 0.105 (NaCl). The nonlinear least squares fit of the data to the Michaelis-Menten equation gave values of k_{cat} = 0.082 s^{-1} and K_m = 130 μM.

Figure S2. Dependence of v_o/[E] (s^{-1}) for the decarboxylation of OMP catalyzed by Q215A mutant yeast OMPDC (11 - 23 nM) on the concentration of OMP in 10 mM MOPS at pH 7.1, 25 ˚C and I = 0.105 (NaCl). The nonlinear least squares fit of the data to the Michaelis-Menten equation gave values of k_{cat} = 21 s^{-1} and K_m = 50 μM.

Figure S3. Dependence of v_o/[E] (s^{-1}) for the decarboxylation of OMP catalyzed by S154A/Q215A mutant yeast OMPDC (3.9 μM) on the concentration of OMP in 10 mM MOPS at pH 7.1, 25 ˚C and I = 0.105 (NaCl). The nonlinear least squares fit of the data to the Michaelis-Menten equation gave values of k_{cat} = 0.042 s^{-1} and K_m = 110 μM.

Figure S4. Timecourses for formation of the product EU from decarboxylation of the truncated substrate EO (7 or 14 mM) catalyzed by S154A mutant OMPDC in 50 mM MOPS at pH 7.1, 25 ˚ C and I = 0.15 (NaCl), monitored by HPLC. The slopes of these plots correspond to the initial velocity of formation of the product, v_o (mM min^{-1}). Key: (●) Reaction of 14 mM EO in the presence of 0.26 mM S154A OMPDC; (○) Reaction of 7 mM EO in the presence of 0.24 mM S154A OMPDC.

Figure S5. Dependence of v_o/[E] (s^{-1}) for the decarboxylation of EO on the initial substrate concentration in 50 mM MOPS at pH 7.1, 25 ˚C and I = 0.15 (NaCl), monitored by HPLC. Key: (■) Data for Q215A mutant yeast OMPDC plotted on the left-hand y-axis; (●) Data for S154A mutant yeast OMPDC plotted on the right-hand y-axis; (◆) Data for S154A/Q215A mutant yeast OMPDC plotted on the right-hand y-axis.

Figure S6. Dependence of the observed second-order rate constant $(k_{cat}/K_m)_{app}$ (M^{-1} s^{-1}) for the decarboxylation of EO on the concentration of added phosphite dianion at pH 7.0, 25 ˚C and I = 0.15 (NaCl), monitored by HPLC. Key: (■) Data for Q215A mutant yeast OMPDC plotted on the left-hand y-axis; (●) Data for S154A mutant yeast OMPDC plotted on the right-hand y-axis; (◆) Data for S154A/Q215A mutant yeast OMPDC plotted on the right-hand y-axis.

FIGURE S1

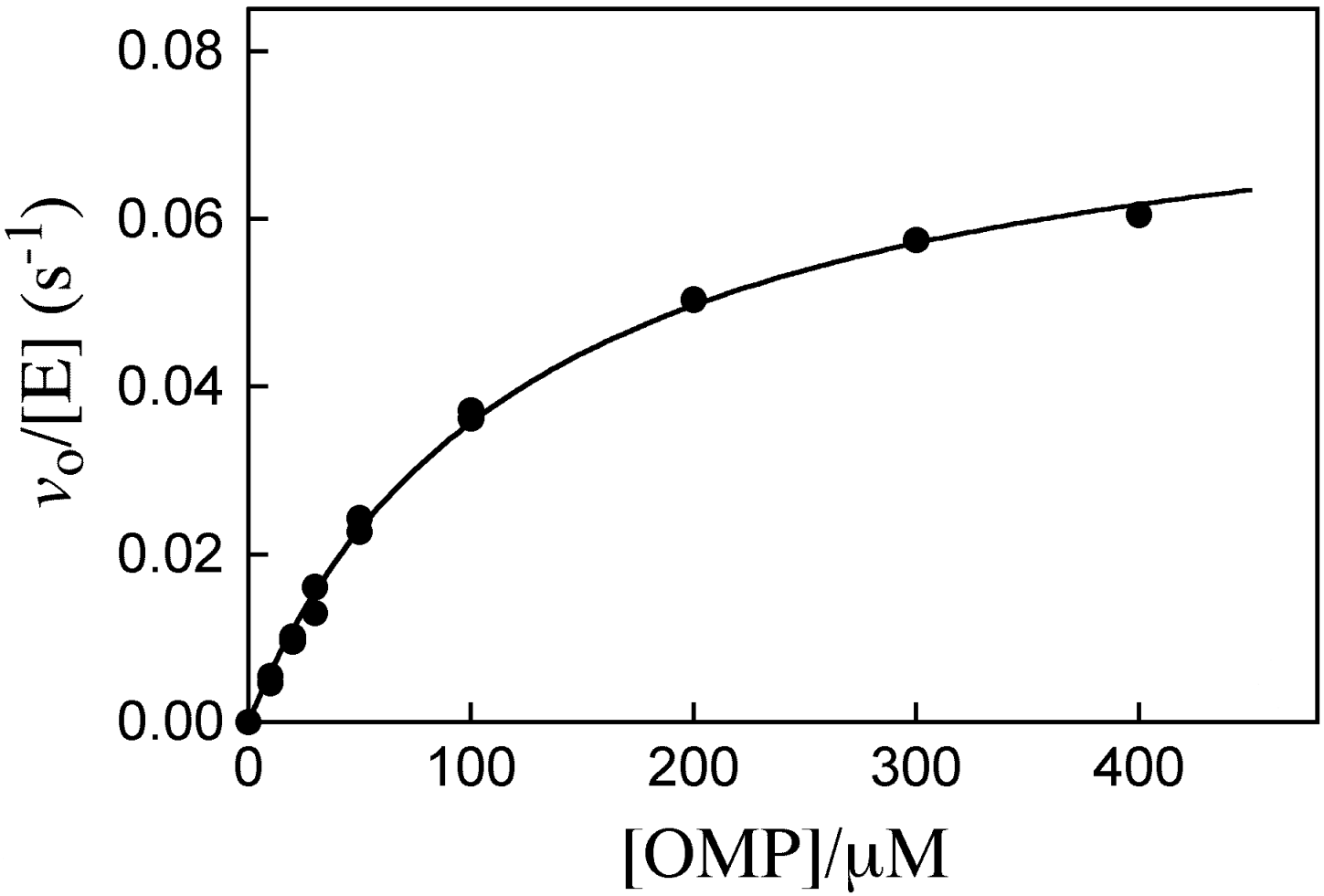

FIGURE S2

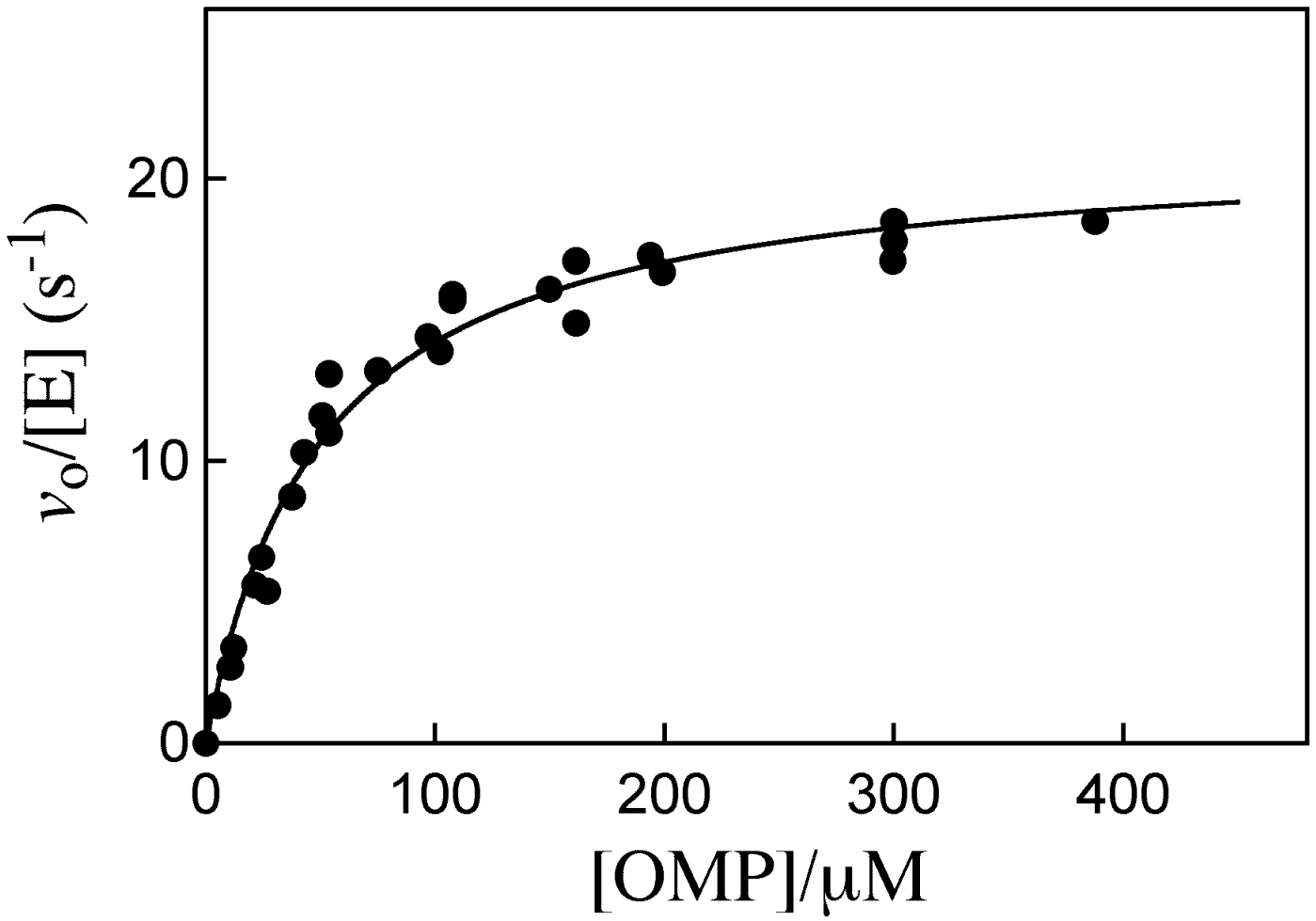

FIGURE S3

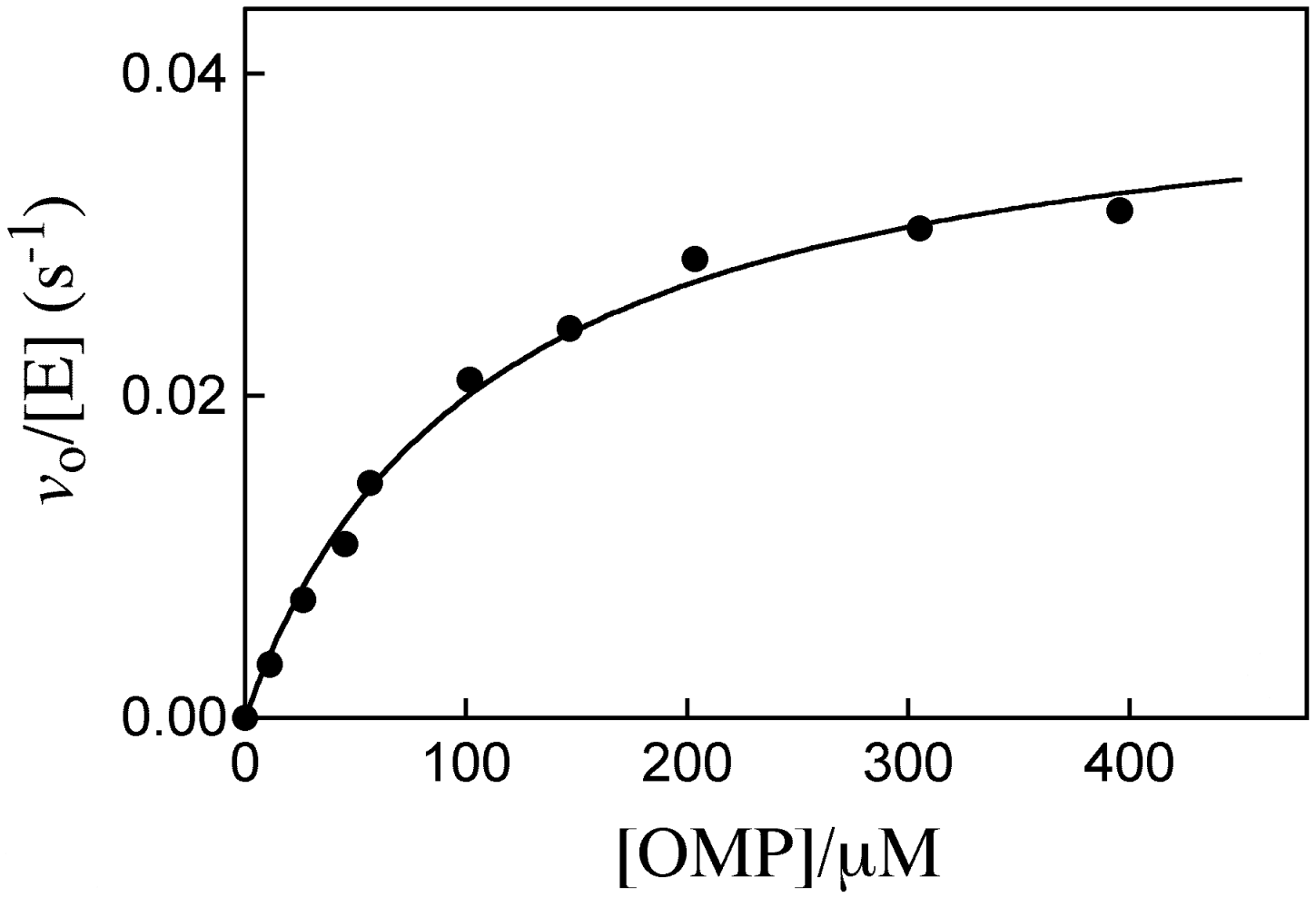

FIGURE S4

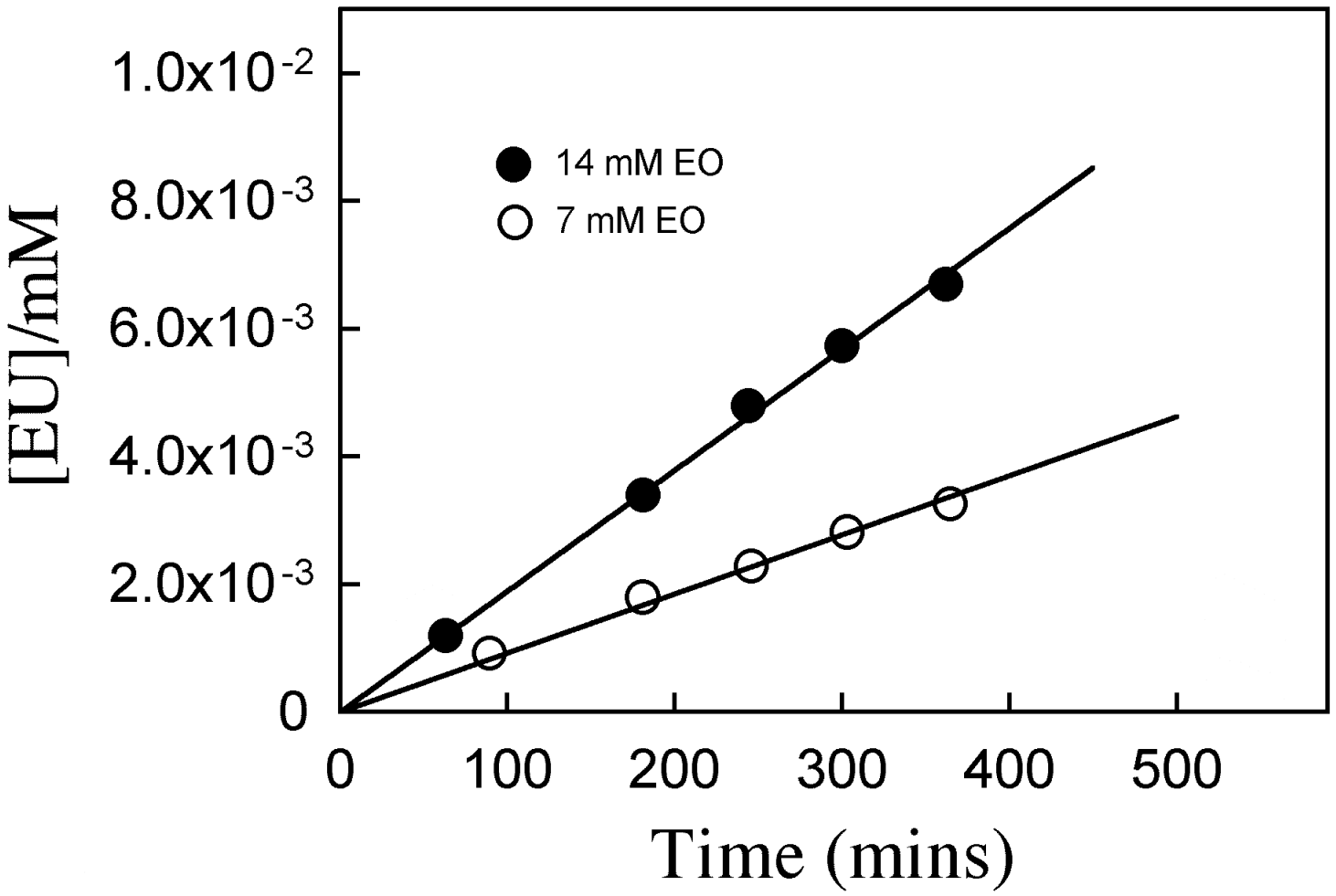
1.0x10-2
8.0x10-3
6.0x10-3
4.0x10-3
2.0x10-3
0
[EU]/mM
14 mM EO
7 mM EO
0
100
200
300
400
500
Time (mins)

FIGURE S5

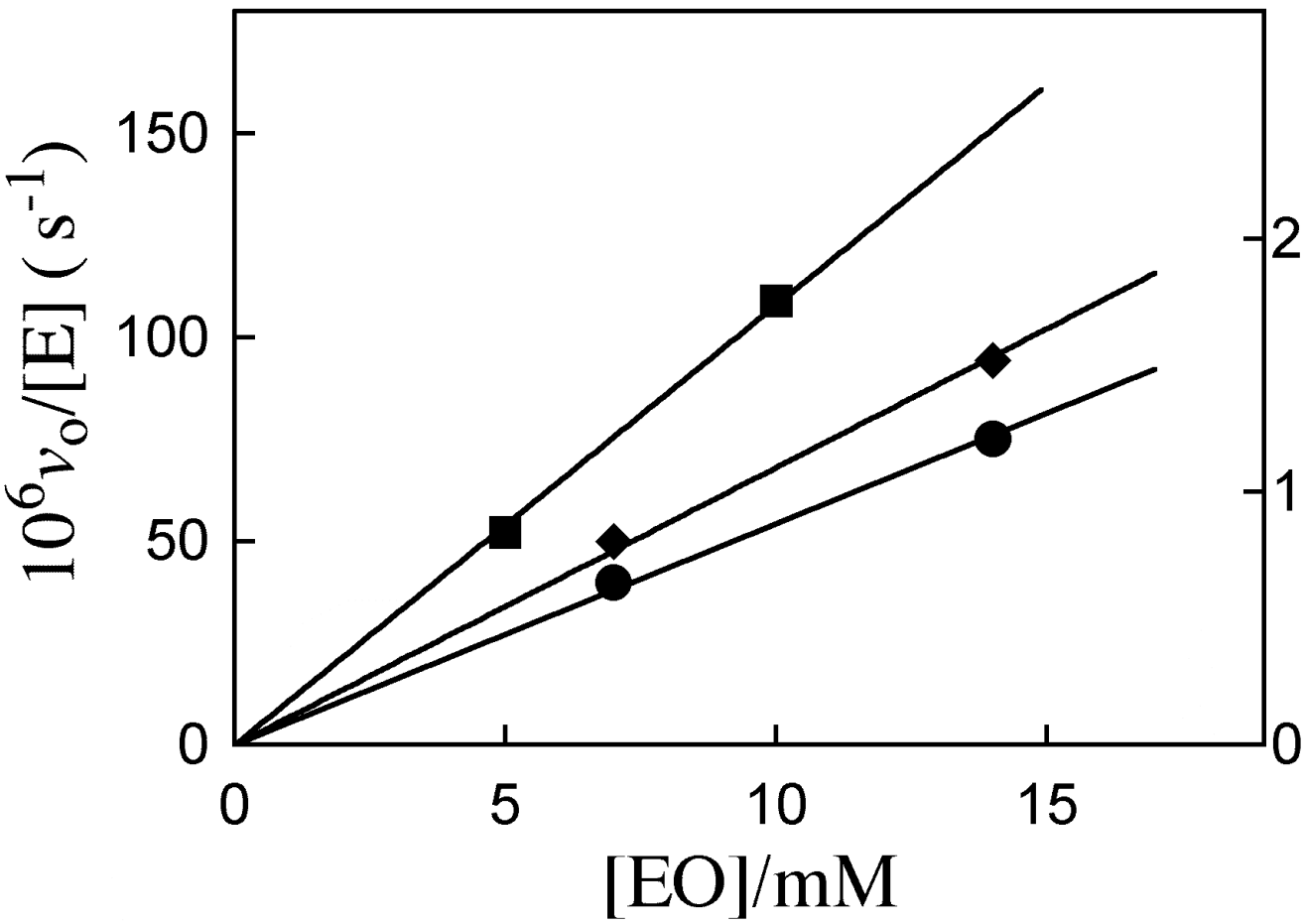

FIGURE S6