# **Supporting Information**

QM/MM Studies of Pyruvate C-C Bond Cleavage by FrsA are not Consistent with its Proposed Function as a Cofactor-Independent Pyruvate Decarboxylase

Whitney F. Kellett,<sup>†</sup> Elizabeth Brunk, Bijoy J. Desai, Alexander A. Fedorov,

John A. Gerlt, Steven C. Almo, Ursula Rothlisberger, and Nigel G. J. Richards $^{\dagger}$ 

Contribution from the Department of Chemistry, University of Florida, Gainesville, FL 32611, USA, the Laboratory of Computational Chemistry and Biochemistry, Ecole Polytechnique Fédérale Lausanne, CH-1015 Lausanne, Switzerland, the Departments of Chemistry & Biochemistry, Institute for Genomic Biology, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA, and the Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY 10461, USA.

<sup>†</sup> Present address: Department of Chemistry & Chemical Biology, 402. N. Blackford. St., Indiana University Purdue University Indianapolis (IUPUI), Indianapolis, IN 46202, USA

## **Obtaining an initial model of the FrsA/pyruvate complex**

Coordinates for the free enzyme monomer were obtained from the PDB (entry: 3MVE) and all crystallographic waters and counter-ions were removed prior to the addition of hydrogen atoms using the tLEAP module of the AMBER11 software package (1, 2). The resulting structure was placed in a box of explicit TIP3P water molecules (3) with two chloride ions to yield a system with zero net charge. After constrained energy minimization, the FrsA structure was carefully equilibrated using a series of constrained molecular dynamics simulations (NPT ensemble). These calculations all employed the AMBER99 force field (4, 5), as implemented in the Sander module of the AMBER11 software package

(1), and the Particle-Mesh Ewald algorithm to treat electrostatic interactions (6). Force field parameters for pyruvate were generated using the Generalized AMBER Force Field (GAFF) (7), and then the GLIDE tool (8) in the MAESTRO package was used to generate models of the FrsA/pyruvate complex. The docking calculations employed an active site consisting of the residues (Arg-53, Asp-203, Arg-272, Phe-273, Phe-303, Tyr-316, Leu-320, Met-338 and Trp-341) proposed in the original description of FrsA structure and function (9). Subsequent energy minimization of eight FrsA/pyruvate complexes followed by constrained MD equilibration (over a period of 40 ns in the NPT ensemble) gave the same final structure, which was then used as the starting point for QM/MM simulations (Figure S1). Coordinates for this FrsA/pyruvate complex in PDB format (10) are available from the authors on request.

**Figure S1** Ribbon representation of the FrsA/pyruvate model complex showing the location of the bound ligand. Pyruvate is rendered as cylinders colored by atom type. Color scheme: C - cyan; H - white; O - red. The figure was produced with the VMD software package (*11*).



## CPMD simulations of pyruvate decarboxylation in the FrsA/pyruvate complex

The QM/MM simulations employed an extension of CPMD (12, 13). Atoms in the QM region, comprising pyruvate, three active site waters, and the Tyr-316 side chain capped at  $C_{\alpha}$  with a monovalent pseudopotential (13), were described by the BLYP functional (14, 15) and norm-conserving Martins-Trouiller pseudopotentials (16) with dispersion-corrected atom-centered potentials (17-20). Wavefunctions were expanded in a plane wave basis set with a 70 Ry cutoff inside an orthorhombic box (37 Å x 22 Å x 22 Å), and long-range interactions were decoupled using the Martyna-Tuckerman scheme (21). The remaining protein atoms and explicit solvent water molecules were described by a classical AMBER99 force field (4, 5). QM/MM simulations were performed at constant pressure and temperature, using a Nosé Hoover thermostat, after slow heating to 300K over 2-3 ps. In these heating simulations, however, the Tyr-316 hydroxyl spontaneously transferred a proton to the pyruvate carboxylate when the temperature exceeded 250 K. Control calculations, using a constraint on the O-H bond of the Tyr-316 moiety, demonstrated that the proton transfer event was not due to the starting configuration. Thus, when the constraint on the O-H bond was released at 300K, spontaneous proton transfer again took place. The resulting system containing neutral pyruvic acid was then equilibrated for 2 ps before constrained MD simulations were performed for thermodynamic integration (22, 23) using a reaction coordinate chosen to be the bond distance between C1 and C2 of the pyruvate molecule. Hence, constraints were employed at distances spanning 1.55 to 4.24 Å (in increments of 25 pm) with sampling at each constrained distance for a period of 1 ps. Although this relatively short time permits only a limited exploration of phase space, the statistical error in the free energy barrier was estimated to be about 0.2 kcal/mol on the basis of the standard deviations in the constraint forces. Moreover, taking only half the sampled points per window gave only a 0.1 kcal/mol change in the calculated barrier, which is smaller than the accuracy expected for standard GGAs. The free energy profile was obtained by integrating the constraint forces over the respective distances.

### CPMD simulations of pyruvate decarboxylation in water

Pyruvate monoanion was placed in a box of explicit TIP3P water molecules (3) with a single potassium ion to yield a system with zero net charge, and the resulting system equilibrated using a series of MD simulations in the NPT ensemble. These calculations all employed the AMBER99 force field (4, 5), as implemented in the Sander module of the AMBER11 software package (1), and the Particle-Mesh Ewald algorithm to treat electrostatic interactions (6). GAFF (7) parameters were employed to describe pyruvate in these equilibration studies. The equilibrated system was then employed in QM/MM simulations (12, 13). Atoms in the QM region comprised the pyruvate monoanion and a water molecule, which was hydrogen bonded to the carboxylate group of pyruvate. These QM atoms were described by the BLYP functional (14, 15) and norm-conserving Martins-Trouiller pseudopotentials (16) with dispersion-corrected atom-centered potentials (17-20). Wavefunctions were expanded in a plane wave basis set with a 70 Ry cutoff inside an orthorhombic box (12 Å x 7 Å x 7 Å), and long-range interactions were decoupled using the Martyna-Tuckerman scheme (21). The remaining water molecules were described by a classical AMBER99 force field (4, 5). QM/MM simulations were performed at constant pressure and temperature, using a Nosé Hoover thermostat, after heating to 300K. After equilibration, constrained MD simulations were performed for thermodynamic integration (22, 23)using a reaction coordinate chosen to be the bond distance between C1 and C2 of the pyruvate molecule. Hence, constraints were employed at distances spanning 1.6 to 4.5 Å (in ten increments) with sampling at each constrained distance for a period of 1 ps. The free energy profile was obtained by integrating the constraint forces over the respective distances.

**Figure S2** Free energy profile (kcal/mol) for cleavage of the pyruvate C-C bond in water, as computed by thermodynamic integration. Error bars show the statistical sum of errors associated with the calculated free energy.



## pKa estimates using Poisson-Boltzmann calculations

The protonation state of all protein residues in the X-ray crystal structure of unliganded FrsA (3MVE), the equilibrated model of the FrsA/pyruvate complex, and several different "frames" from classical MD trajectories for unliganded FrsA and the FrsA/pyruvate complex were calculated (Table S1) using two well-established methods, H++ (24, 25) and Adaptive Poisson-Boltzmann (APB) (26). These calculations suggest that the  $pK_a$  of the Tyr-316 side chain is lowered by approximately one unit when pyruvate is bound to the protein (Table S1). Superimposing different MD snapshots of the active site is consistent with the view that electrostatic repulsion between the substrate carboxylate and the Asp-203 side chain, and steric interactions with Met-312, positions pyruvate close to Tyr-316 and Arg-273 (Figure S3).

**Table S1**  $pK_a$  values computed for Tyr-316 in unliganded FrsA (3MVE) and various MD trajectory "snapshots" of the FrsA/pyruvate complex, and for pyruvate in the final model of the FrsA/pyruvate complex.

Residue	Calculated pKa (Method A) <sup>a</sup>	Calculated pKa (Method B) <sup>a</sup>
Tyr-316 (3MVE)	9.6	10.5
Tyr-316 (FrsA/pyruvate: 18.60 ns)	9.8	9.9
Tyr-316 (FrsA/pyruvate: 18.75 ns)	9.9	9.9
Tyr-316 (FrsA/pyruvate: 18.90 ns)	9.0	10.1
Tyr-316 (FrsA/pyruvate: 19.00 ns)	9.2	9.2
Pyruvate (FrsA/pyruvate: 19.00 ns)	n.d.	3.8
<sup>a</sup> H <sup>++</sup> ( <u>http://biophysics.cs.vt.edu/H</u> ++	-)	

<sup>b</sup> APBS (ref. 26)

**Figure S3** Superimposed snapshots of the active site from the MD trajectory of the equilibrated FrsA/pyruvate complex. The average distance between pyruvate and the Asp-203 side chain is shown by the dashed line. Residue coloring reflects the time of the snapshot, ranging from red (early) to blue (late).



#### Protein expression and purification

## Method A

The gene encoding the FrsA from Vibrio vulnificus in the plasmid pOE-FrsA was generously provided by Dr. Sun-Shin Cha (Marine Biotechnology Research Center, Korean Ocean Research & Development Institute) and expressed in *Escherichia coli* JM109 cells containing the F' episomal element, as reported previously (9). F' is essential for protein expression, and JM109 cells containing this element were identified by screening on minimal growth media containing proline. After transformation with pQE-FrsA, starter cultures of JM109 cells were grown overnight in M9 minimal media supplemented with thiamin (5  $\mu$ g/mL), L-proline (5  $\mu$ g/mL) and ampicillin (100  $\mu$ g/mL). These starter cultures were used to inoculate 3 L of M9 minimal media supplemented with thiamin, L-proline and ampicillin (100 µg/mL). The cultures were grown with aeration at 37 °C to mid-log phase (O.D of 0.6) and then heat shocked at 42 °C for 15 min before the addition of IPTG (0.5 mM) to induce protein expression. Cells were harvested after 6 h, lysed using sonication, and subsequently centrifuged at 5000x g to obtain a clear lysate that was added to Ni-NTA resin suspended in a phosphate wash buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>) containing 10 mM imidazole and 500 mM KCl, pH 7). After 1 h incubation on ice, FrsA was eluted from the resin (50 mM NaH<sub>2</sub>PO<sub>4</sub> containing 250 mM imidazole and 500 mM KCl, pH 7) and proteincontaining fractions were pooled and dialyzed into 50 mM NaH<sub>2</sub>PO<sub>4</sub> containing 500 mM KCl and 1 mM DTT, pH 7. The inclusion of DTT in the elution buffer was to prevent formation of the FrsA dimer by a disulfide bond between the cysteine residues, which had been reported to be inactive (9).

### Method B

The codon-optimized gene encoding the FrsA from *Vibrio vulnificus* was synthesized (GenScript, Piscataway NJ) and ligated into the PUC57 plasmid with NdeI and BamHI restriction sites at the 5' and 3' ends, respectively. The resulting plasmid was subjected to digestion with NdeI and BamHI and ligated into the pET15b expression vector (N-terminal His<sub>6</sub>-tag). The ligated plasmid was transformed

into *Escherichia coli* XL1 blue cells, and a single colony was inoculated into 5 mL of LB containing ampicillin ( $100\mu g/mL$ ) and allowed to grow overnight at 37 °C. The plasmid encoding FrsA was then transformed into *Escherichia coli* BL21 (DE3) cells and starter cultures prepared from single colonies. These were used to inoculate 4 L of LB containing ampicillin ( $100 \mu g/mL$ ), and resulting cultures were grown at 37 °C with aeration to mid-log phase (O.D. ~ 0.6) before the addition of IPTG (1 mM final concentration). Cells were harvested after 4 h, and lysed by sonication. The clarified lysate was then loaded onto Ni-NTA resin and FrsA eluted using an imidazole gradient. The fractions containing FrsA (determined by SDS PAGE) were pooled, dialyzed against phosphate buffered saline containing 3 mM DTT, and the His-tag cleaved using thrombin (Promega, Madison WI). The resulting preparation of FrsA was dialyzed against storage buffer (20 mM HEPES containing 150 mM NaCl and 3 mM DTT, pH 7.5). After concentration to ~ 10 mg/mL, the purified FrsA was stored in 20  $\mu$ L aliquots at -80 °C.

## Kinetic assays of recombinant, wild type FrsA

# Method A: Membrane-Inlet Mass Spectrometry (MIMS) for direct detection of CO2

The real-time production of <sup>13</sup>CO<sub>2</sub> was monitored by MIMS (*27*) when 1 mM <sup>13</sup>C-labeled pyruvate was incubated with 50 nM recombinant FrsA dissolved in 50 mM NaH<sub>2</sub>PO<sub>4</sub> buffer, pH 7, containing 500 mM KCl and 1 mM DTT, over a period of 12 min at 37°C. No detectable decarboxylase activity was observed.

*Method B: Spectrophotometric assay for pyruvate decarboxylation*. Decarboxylation of pyruvate to acetaldehyde was measured using a coupled assay with yeast alcohol dehydrogenase (Sigma-Aldrich, St. Louis MO) by monitoring the decay in NADH absorbance at 340 nm. Assays were carried out in 50 mM sodium phosphate, pH 7.0; containing 1 mM DTT; 5 mM pyruvate; 0.2 mM NADH; 3.7 units of alcohol dehydrogenase and FrsA ranging from 1 nM to 1 uM. No detectable activity was observed. At the end of each assay, acetaldehyde was added to ensure that the alcohol dehydrogenase was active.

*Method C:* <sup>1</sup>*H NMR detection of the FrsA-catalyzed pyruvate decarboxylation.* <sup>1</sup>*H NMR spectroscopy* was used to detect acetaldehyde formation when 5  $\mu$ M FrsA was incubated for 90 min at 20 °C with 10 mM pyruvate in D<sub>2</sub>O with 50 mM phosphate, pD 7.0, containing 1mM DTT. Although signals corresponding to the methyl group protons of unhydrated and hydrated pyruvate anion were detected, no resonance(s) were detected for the protons in acetaldehyde.

#### Crystallization, data collection, structure determination and crystallographic refinement

Crystals of recombinant, wild type FrsA were grown at room temperature using the "sitting drop" method. In these experiments, the protein solution contained FrsA (20 mg/mL), 150 mM NaCl and 3 mM DTT dissolved in 20 mM Hepes buffer, pH 7.5 and the precipant was composed of 20% PEG monomethyl ether 2000 and 200 mM trimethylamine N-oxide in 0.1 M Tris buffer, pH 8.5. Crystals appeared in 3-4 days and exhibited diffraction consistent with the space group P2<sub>1</sub>, with two polypeptides per asymmetric unit. Prior to data collection, the crystals of FrsA were transferred to cryoprotectant solutions, composed of their mother liquids containing 20% glycerol, and flash-cooled in a nitrogen stream. The X-ray diffraction data set (Table S2) was collected at the NSLS X29A beamline (Brookhaven National Laboratory) on the 315Q CCD detector. Diffraction intensities were integrated and scaled with programs DENZO and SCALEPACK (*28*).

The FrsA X-ray crystal structure (Figure S4) was solved using only input diffraction and sequence data by molecular replacement with the fully automated molecular replacement pipeline BALBES (29). The search model employed by BALBES was that of a previously reported structure for FrsA (3MVE) (9). After obtaining a partially refined structure from BALBES, several iterative cycles of refinement were then performed. Thus, model rebuilding was performed with COOT (30) and refined with PHENIX (31), followed by automatic model rebuilding using the ARP software package (32). The quality of the final structure was verified with omit maps, and stereochemistry checked with WHATCHECK (33) and

MOLPOBITY (*34*). All loops are well defined in the current FrsA structure, with only the backbone torsion angles of Asn-108 adopting values in the disallowed region of the Ramachandran plot. The program LSQKAB 35) was used for structural superposition, and figures with electron density maps were prepared using PYMOL. Final crystallographic refinement statistics are shown in Table S2.

Table S2 Crystallographic data collection and refinement statistics.<sup>a</sup>

**Data Collection** 

Resolution (Å)	∞-1.95
X-ray Source	NSLS X29A
Wavelength (Å)	1.075
Space Group	P2.
Molecules in asymmetric unit	2
Cell dimensions	a = 42.66 Å $b = 104.54$ Å $c = 82.30$ Å
Cell dimensions	a = 42.00  M, b = 104.04  M, c = 62.00  M, c =
Unique reflections observed	p = 91.10 51262
	07.9
Completeness (%)	97.8
K <sub>merge</sub>	0.083
Refinement	
Protein residues/water atoms per asu	6405/274
Other ligands per asu	2
Reflections (work/free)	51363/2608
$R_{cryst}/R_{free}$ (%)	17.9/22.7
Resolution (Å)	25.00-1.95
Average B-factor $(Å^2)$	29.2
Protein $(Å^2)$	29.3
Hexanoic acid $(Å^2)$	32.9
PEG monomethyl ether $(Å^2)$	47.5
$RMSD^{c}$ Bond lengths (Å)	0.008
<b>RMSD</b> Bond angles $\binom{0}{1}$	1.05
Riviob Dona angles ()	1.05

<sup>a</sup> Coordinates are deposited as PDB entry 4I4C.

<sup>b</sup>  $R_{merge} = \sum_{hkl} \sum_{i} |I_{hkl, i} - \langle I_{hkl} \rangle |I| / \sum_{hkl} \sum_{i} |I_{hkl, i}|$ , where  $\langle I_{hkl} \rangle$  is the mean intensity of the multiple  $I_{hkl, i}$  observations for symmetry-related reflections.

<sup>c</sup> RMSD: root mean square deviation.

**Figure S4** Ribbon representation of the FrsA monomer containing putative ligands. The N- (1-165) and C-terminal (166-415) domains are colored brown and cyan, respectively. Bound PEG monomethyl ether (H<sub>3</sub>C[OCH<sub>2</sub>CH<sub>2</sub>]<sub>5</sub>OH) and hexanoic acid (H<sub>3</sub>CCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub> COOH) ligands are rendered as cylinders colored yellow and green, respectively, except for oxygen atoms, which are shown in red. Bound hexanoate is positioned in a very similar manner to pyruvate in the equilibrated computational model of the FrsA/pyruvate complex (Figure S1). The image was produced with PYMOL (DeLano Scientific Software LLC, Palo Alto, CA).



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