THE REACTIVE FORM OF A C-S BOND-CLEAVING CO$_2$-FIXING FLAVOENZYME

by

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# TABLE OF CONTENTS

1. OVERVIEW OF CARBOXYLASES ................................................................. 1
   Significance .................................................................................................. 1
   The Challenge of CO$_2$ ........................................................................... 1
   Representative Carboxylases .................................................................. 2
      RuBisCO ................................................................................................. 2
      PEP-C ..................................................................................................... 3
      ACC ......................................................................................................... 4
      2-KPCC .................................................................................................... 4
   Typical DSORs vs 2-KPCC ...................................................................... 5
      Glutathione reductase compared to 2-KPCC ....................................... 5
      Thioredoxin reductase compared to 2-KPCC ....................................... 7

2. EXPERIMENTAL ......................................................................................... 8
   Expression and purification of wt and mutant 2-KPCC ........................... 8
   Reductive Half reaction measured by diode array and photomultiplier detection .. 9
   Measurement of K$_D$ for NADP$^+$ by fluorescence titration .................. 10

3. THE REACTIVE FORM OF 2-KPCC ............................................................ 11
   Introduction ............................................................................................... 11
   Results ......................................................................................................... 11
      The rate of 2-KPCC reduction by NADPH at low concentrations .......... 11
      Characterization of the reductive half reaction ................................... 12
      The reductive half reaction using single wavelength excitation and photomultiplier detection ......................................................... 13
      WT vs C82A multi-site binding of NADP$^+$ ....................................... 14
      Addition of an active site proton donor residue in F501H .................... 15
      Summary of CO$_2$-fixing flavoenzyme .............................................. 16
      Conclusions ............................................................................................. 19

REFERENCES CITED ..................................................................................... 32

APPENDIX ...................................................................................................... 35
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Relative speciation and reactions of activation of carbon dioxide</td>
<td>20</td>
</tr>
<tr>
<td>2.</td>
<td>Overview of RuBisCO reactions</td>
<td>21</td>
</tr>
<tr>
<td>3.</td>
<td>Overview of representative carboxylases</td>
<td>22</td>
</tr>
<tr>
<td>4.</td>
<td>Canonical steps by which the active site of DSORs are reduced, yielding the reactive form of the active site</td>
<td>23</td>
</tr>
<tr>
<td>5.</td>
<td>Proposed oxidative half reaction for 2-KPCC under carboxylating conditions</td>
<td>24</td>
</tr>
<tr>
<td>6.</td>
<td>The rate of 2-KPCC reduction by NADPH saturates at low concentrations</td>
<td>25</td>
</tr>
<tr>
<td>7.</td>
<td>The slow reductive half reaction of 2-KPCC has flavin reduction and reoxidation steps followed by photoreduction</td>
<td>26</td>
</tr>
<tr>
<td>8.</td>
<td>The reductive half reaction in 2-KPCC is slow relative to typical DSORs and occurs in multiple observable phases</td>
<td>27</td>
</tr>
<tr>
<td>9.</td>
<td>Possible protonation states for the glutathione reductase and 2-KPCC active sites</td>
<td>28</td>
</tr>
<tr>
<td>10.</td>
<td>Wild type and C82A (C82 = Cys\text{\textsubscript{INT}}) 2-KPCC show multisite binding of NADP\textsuperscript{+} with high affinity</td>
<td>29</td>
</tr>
<tr>
<td>11.</td>
<td>Addition of an active site proton donor residue in F501H has no impact on the rate of 2-KPCC reduction by NADPH</td>
<td>30</td>
</tr>
<tr>
<td>12.</td>
<td>Pre-reduced 2-KPCC forming an irreversible crosslink with suicide inhibitor BES</td>
<td>31</td>
</tr>
</tbody>
</table>
Atmospheric carbon dioxide (CO$_2$) is used as a carbon source for building biomass in plants and most engineered synthetic microbes. Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), the most abundant enzyme on earth, is used by these organisms to catalyze the first step in CO$_2$ fixation. Microbial processes that also fix carbon dioxide or bicarbonate have more recently been discovered. My research focuses on a reaction catalyzed by 2-KPCC (NADPH:2-ketopropyl-coenzyme M oxidoreductase/carboxylase), a bacterial enzyme that is part of the flavin and cysteine-disulfide containing oxidoreductase family (DSORs) which are best known for reducing metallic or disulfide substrates. 2-KPCC is unique because it breaks a comparatively strong C-S bond, leading to the generation of a reactive enolacetone intermediate which can directly attack and fix CO$_2$. 2-KPCC contains a phenylalanine in the place where most other DSOR members have a catalytically essential histidine. This research focuses on studying the unique reactive form of 2-KPCC in presence of an active site phenylalanine.
OVERVIEW OF CARBOXYLASES

Significance

This research will lead to greater understanding of a novel carboxylation reaction that will help clarify research into microbial contributions to the carbon cycle. Furthermore, this research could support the development of future strategies for carbon dioxide capture and fixation. This will lead to a more fundamental understanding of carboxylation chemistry that can potentially be used to help combat climate change. It will also help us better understand the global importance of microbes as a sink for CO₂.

The Challenge of CO₂

Carbon dioxide fixation presents three chemical challenges to an enzyme. The first is that CO₂ has to be trapped and bound to the enzyme. CO₂ is an uncharged molecule that is in low natural abundance, with less than 1% of the atmosphere (i.e., approximately 0.04%) consisting of CO₂. However, CO₂ has a relative speciation which is dependent on pH. In solution below pH 6 the dominant form will be CO₂, while between pH 7 and pH 10 the dominant form will be bicarbonate, and above pH 10 it is carbonate (Figure 1a). Therefore, at physiological pH the typical enzymatic substrate is bicarbonate, which is usually dehydrated and either phosphorylated by ATP or affixed to a biotin cofactor (Figure 1b, 2c). This allows CO₂ to be transported and later released from the phosphoryl-group or biotin moiety. The second challenge to fixing CO₂ is to generate a nucleophilic carbon located near the bound CO₂ in the active site (Figure 1d). The final challenge is to direct
the nucleophilic carbon to attack CO₂ instead of a more abundant and more reactive molecules, such as oxygen or hydrogen.¹,²,⁵ All of these characteristics make CO₂ a challenging enzyme substrate.

Representative Carboxylases

Carboxylases are a class of enzymes that catalyze the net fixation of carbon dioxide or bicarbonate into an organic substrate. All carboxylases generate a nucleophilic carbon near a chemical bond.¹ Though all carboxylases catalyze this reaction, this is achieved through a variety of mechanisms that have substantial differences relating to co-substrate, cofactor, or metal requirements. In general, biological carboxylation requires the action of biotin or a divalent metal cofactor, and it requires ATP when the carboxylation agent is the bicarbonate ion.¹,⁵ Here I will focus on some representative carboxylase enzymes and how they perform carbon dioxide fixation, and then I will compare these to the novel mechanism of 2-KPCC.

Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase

RuBisCO catalyzes the carboxylation step found in photosynthesis.¹ Photosynthesis can be broken into two stages: light-dependent and light-independent reactions. The light-independent reactions are referred to as the Calvin cycle, which utilize the products of the light reaction, ribulose-1,5-bisphosphate (RuBP) and carbon dioxide, to generate glyceraldehyde 2-phosphate (Figure 3a).² To perform this reaction, RuBisCO must be first activated by a single one-time reaction with carbon dioxide.⁶ CO₂ binds the active site residue Lys201 and makes a carbamyl group that coordinates the Mg²⁺ ion and
causes a conformational change to make a catalytically ready active site. Once this occurs the enzyme is active and can coordinate and bind RuBP and atmospheric CO$_2$ at the Mg$^{2+}$ cofactor active site. RuBisCO also undergoes a side reaction with oxygen to make phosphoglycolate, which is not energetically favorable to the cell. This side reaction activity comes from the reactive enediolate intermediate. It is theorized that in early stages of evolution for plants, that O$_2$ was not present or very low concentration in the atmosphere, so the side reaction was not a competing factor. RuBisCO discriminates between CO$_2$ and O$_2$ through the hydrophilic residues in its active site, which help bind CO$_2$ and exclude O$_2$. (Figure 2)

**Phosphoenolpyruvate Carboxylase**

Phosphoenolpyruvate carboxylase (PEP-C) is an enzyme that was evolutionarily developed to address the needs of plants that live in hot climates (i.e. the desert), this enzyme plays a critical role in C-4 photosynthesis. PEP-C uses bicarbonate as its enzymatic substrate. The overall reaction is as follows, Mg$_2^+$ binds first, followed by phosphoenolpyruvate and then followed by bicarbonate, a reaction intermediate commonly known as carboxyphosphate is formed, followed by the formation of enolpyruvate and then finally the product oxaloacetate (Figure 3b). This dehydration of bicarbonate is unique because it is a biotin independent reaction, carboxyphosphate is an unstable intermediate with a lifetime of ~70ms, so the reaction must happen quickly and produce carbon dioxide in the active site to carboxylate the substrate.
**Acetyl CoA Carboxylase**

Acetyl Coenzyme A Carboxylase (ACC) is a biotin and ATP dependent enzyme found in fatty acid synthesis. The reaction starts with ACC using a biotin cofactor to dehydrate bicarbonate and create a carboxybiotin intermediate. The intermediate is then transferred to acetyl CoA and forms malonyl CoA (Figure 3c). This enzyme functionalizes bicarbonate in a way that is considered the standard way for an enzyme, which is to use an organic cofactor, biotin, in order to dehydrate bicarbonate. This is considered the standard way for functionalizing bicarbonate because a carboxybiotin intermediate is a lot more stable than a carboxyphosphate intermediate (as mentioned in the previous section), and therefore less likely to degrade during reaction.

**NADPH: 2-ketopropyl coenzyme M oxidoreductase/carboxylase as a carboxylase**

2-KPCC (NADPH: 2-ketopropyl coenzyme M oxidoreductase/carboxylase) is unique compared to other carboxylases in a variety of ways. 2-KPCC lacks biotin, metals, and ATP, which other enzymes previously discussed utilize in order to use carbon dioxide or bicarbonate as a substrate. Instead, 2-KPCC employs a flavin cofactor, NADPH, and an active site disulfide bond in order to trap and carboxylate carbon dioxide into acetoacetate. This unique reaction mechanism comes from an enzyme found in a bacterial pathway for alkene epoxidation. The enzyme is part of a group of flavin and cysteine containing oxidoreductases (DSORs), that are known for reducing metallic or disulfide substrates.
Typical Flavin and Cysteine containing Oxidoreductases (DSORs) compared to 2-KPCC

Comparing some key DSOR family members to 2-KPCC can help us understand the mechanism of 2-KPCC. 2-KPCC is unique in its family because it is the only one to catalyze a carboxylation reaction. 2-KPCC has a phenylalanine in the place of a catalytically conserved histidine, which most other members of its family, such as glutathione reductase share.

Glutathione Reductase compared to 2-KPCC

Glutathione reductase catalyzes the reduction of glutathione disulfide by NADPH. As a member of the flavin and cysteine containing disulfide family, it is homodimeric and has a redox active disulfide and an FAD cofactor in each monomer. All DSORs share a common reaction mechanism which can be broken into two halves, reductive and oxidative. In the slower reductive half reaction, the active site is reduced, generating the active form that is used in the oxidative half. The reductive half starts with the transfer of the hydride of the NADPH to the FAD cofactor, then to the active site disulfide. This generates the reduced active site as a doubly protonated form (EH$_2$), where the cysteine distal to the FAD (known as the interchange thiol, Cys$\text{INT}$) is protonated, and the cysteine closest to the FAD cofactor (known as the charge transfer thiol, Cys$\text{CT}$) participates in a charge transfer interaction with the FAD. This interaction is stabilized by the charge on the active site acid-base histidine (HisH$^+$)(Figure 4, species 6). Thus the enzyme is now poised to reduce a substrate in the oxidative half reaction, where the glutathione disulfide (GSSG) is reductively cleaved by Cys$\text{INT}$ and protonated by HisH$^+$ to form one molecule of glutathione (GSH) and a covalent, mixed disulfide intermediate. Protonation of that
intermediate then yields a second GSH and allows for the oxidized cysteine disulfide to reform\(^{11}\) (Figure 4). There have been mutagenesis studies of glutathione reductase where the active site histidine has been replaced with alanine (H439A),\(^{11-13}\) yielding reaction kinetics much slower (~2 orders of magnitude slower) than wild type (Figure 4). This suggests that the histidine plays a key role in the reaction of glutathione reductase.

2-KPCC shares a common mechanism with glutathione reductase that can be divided into two half reactions. As in glutathione reductase, the slower reductive half of 2-KPCC transfers the hydride of NADPH to a conserved cysteine disulfide through the FAD cofactor. However, 2-KPCC lacks the conserved histidine found in glutathione reductase and in its place has a phenylalanine. Because of this, the steps of the mechanism of reduction and oxidation proceed in a different fashion than described for glutathione reductase, and 2-KPCC has a unique catalytically active form. This is supported by the reductive half reaction proceeding significantly slower (~10 orders of magnitude slower) in 2-KPCC than in glutathione reductase (Figure 4), with kinetics much more similar to the H439A mutant of glutathione reductase.\(^{11,13}\) The reduced reactive form of 2-KPCC can then reductively cleave a substrate. However, instead of a disulfide substrate, 2-KPCC cleaves a strong C-S bond of a thioether substrate known as 2-KPC (2-ketopropryl-coenzyme M, or [2-(2-ketopropylthio)ethanesulfonate]). The first product of this cleavage is an unstable enolacetone anion which nucleophilically attacks enzyme-bound CO\(_2\) and forms acetoacetate. In the absence of CO\(_2\) and in the presence of available protons, the protonation product, acetone, forms in an unwanted side reaction (Figure 6). 2-KPCC is
thus an interesting and unique enzyme which has repurposed the oxidoreductase model to catalyze a carboxylation following a reductive bond cleavage.

**Thioredoxin Reductase compared to 2-KPCC**

Thioredoxin reductase is another enzyme found in the flavin and cysteine disulfide containing family. While most enzymes have essentially the same structure and mechanism regardless of enzyme origin (i.e. if found in prokaryote, archaea, eukaryote). There are two distinct types of thioredoxin reductase. A high molecular weight thioredoxin reductase isolated from *Plasmodium falciparum* and other higher eukaryotes, and a lower molecular weight thioredoxin reductase found in *E. coli*. Both are dimeric, and catalysis happens via FAD and a redox active disulfide, however, there are significant differences between the mechanism of the higher and lower molecular weight varieties, with the higher molecular weight having a third redox active group in each subunit. For the purpose of comparing thioredoxin reductase to 2-KPCC, an evaluation to the lower molecular weight thioredoxin reductase will be made.

Thioredoxin reductase in *E. coli* catalyzes the NADPH-dependent reduction of thioredoxin. Like glutathione reductase the reaction mechanism has reductive and oxidative halves, and breaks a disulfide bond. However, thioredoxin reductase does not have a catalytically active histidine and instead has a buried aspartate near the active site. Due to the lack of a histidine residue, thioredoxin reductase has a distinctive protonated active form, wherein the thioredoxin reductase active site cysteine residues share a proton. The lack of catalytically active histidine makes thioredoxin reductase similar to 2-KPCC and a good model mechanism comparison.
EXPERIMENTAL PROCEDURES

Expression and Purification of WT and mutant 2-KPCC

_E. coli_ BL21 (DE3) pLysS cells were transformed with the pBAD plasmid containing the WT or corresponding mutant 2-KPCC gene from _Xanthobacter autotrophicus_ Py2, plated on LB agar + kanamycin (25 μg/mL) and grown overnight in a 37° incubator. A single colony from the plate was used to grow a 50 mL overnight culture in LB. The 50 mL culture was used to inoculate six, 2L flasks baffled flasks containing 500 mL of ZYP-Rich media and kanamycin (25 μg/mL). Cells were grown at 37°C with agitation at 225 rpm until the OD<sub>600</sub> reached 0.6-1.0. The temperature was reduced to 25°C, arabinose was added to 0.02%, and the cells were grown for an additional 16-18 hours. Cells were pelleted by centrifugation, frozen, and stored at -80°C.

The cell pellet was resuspended in 4 volumes of Lysis Buffer (20mM Tris pH 8.0, 300 mM NaCl, 5 mM imidazole, 1 mM PMSF) and thawed at 30 °C. All subsequent treatments were performed on ice or at 4 °C. Resuspended cells were lysed via multiple rounds of sonication (Branson Ultrasonifier). Cell lysates were clarified via centrifugation at 95,000 x g for 45 min. Clarified lysates were loaded onto a Ni-NTA resin column via gravity, washed with Lysis Buffer, and eluted using a 0-0.4 M imidazole gradient in Lysis Buffer at 2 mL/min. The eluted 2-KPCC was diluted 5-fold into Buffer A (20 mM Tris-Cl pH 6.5, 5% w/v glycerol), applied to a DEAE-sepharose ion-exchange column (GE biosciences), and eluted using a 0-1M NaCl gradient in Buffer A. Fractions were screened using SDS-PAGE. Pure 2-KPCC protein was pooled and dialyzed in 20 mM Tris-Cl pH
7.4, 10% glycerol and 200 mM NaCl. Protein was pressure concentrated using 10 kDa MWCO filters (Millipore). Total protein concentration was determined using a BCA protein assay (Thermo Scientific) while flavin concentration was determined from its UV-vis absorbance at 450 nm using an $\varepsilon_{450} = 11,828 \text{ M}^{-1}\text{cm}^{-1}$. All concentrations of protein cited in the text refer to flavin-containing protein. Procedure found in Streit et al 2019.

**Reductive Half reaction monitored by diode array and photomultiplier detection**

Data were measured using a KinetAssyst stopped flow spectrophotometer (Hi-Tech Scientific) in single-mixing mode with either diode array or single wavelength detection at 460 nm. The spectrometer was made anaerobic using 2mM sodium dithionite solution and then removed using deoxygenated buffer. All stock solutions of 2-KPCC were made anaerobic using a double-manifold schlenk line with alternating cycles of argon gas purging and evacuation and placed in an anaerobic chamber (COY). For monitoring the reductive half reaction with NADPH, solutions of 2-KPCC were diluted into working concentrations into 100mM KPi , 200 mM NaCl, pH 6-7.5 or 100 mM Glycine-NaOH, 200 mM NaCl, pH 8-9.5. 15 µM 2-KPCC was then sealed in an airtight tonometer that interfaced with the stopped flow. Deoxygenated buffer or NADPH solutions were prepared in the anaerobic chamber, sealed in gastight syringes and then introduced to the sample handling unit manifold of the stopped flow for reaction. Data were measured at varying time points and wavelengths and fit using the kinetic studio (Hi-Tech Scientific) software to single or double exponential functions to determine rate constants. For each experimental condition, all data were measured in at least triplicate and averaged.
Measurement of $K_D$ for NADP+ by fluorescence titration

Fluorescence excitation at 460 nm of 2-KPCC gives rise to large fluorescence band between 480-600 nm, with a maximal signal at 525 nm. Titration of 5 μM WT, CysINTAla, or CysINTAla/F501H (100 mM Phosphate buffer pH 6.5 or 100 mM CAPS buffer pH 9.5) with NADP+ resulted in loss of the fluorescence signal at 525 nm. Plots of the change in fluorescence intensity ($\Delta$fluor) versus NADP+ concentration were indicative of multi-site binding and were fit with a sum of two Langmuir isotherms to determine $K_d$ values:

$$\Delta \text{fluor} = \frac{[L]}{K_{D1}+[L]} + \frac{[L]}{K_{D2}+[L]}$$

Where $[L]$ is the free (unbound) ligand concentration. Procedure found in Streit et al 2019.
THE REACTIVE FORM OF 2-KPCC

Introduction

Examples of natural and engineered enzymes that directly functionalize atmospheric $\text{CO}_2$ in the presence of strong electrophiles that can compete with $\text{CO}_2$ are rare. We have tested a mechanism for $\text{CO}_2$ mediated carboxylation by 2-KPCC in the absence of biotin, metals, or ATP. Additionally, 2-KPCC is a part of a flavin and cysteine containing oxidoreductase family. Above I have outlined some key similarities and differences compared to two members (glutathione and thioredoxin reductase) yet, 2-KPCC is the only member of this family to catalyze a carboxylation. Because of this, the steps of reduction might lead to a different reduced form of the active site than in other DSORs, thereby poising the active site to cleave a thioether bond. Here my contributions to the published paper Streit et al 2019 in *Journal of Biological Chemistry* will be presented.

Results

The Rate of 2-KPCC Reduction by NADPH at Low Concentrations

Looking at the reductive half reaction as a function of NADPH concentration showed that the rate of reductions by NADPH saturates at very low levels, with the rate constants reaching a saturating value by 150 $\mu$M NADPH (Figure 6). Using this information the reductive half reaction was monitored using saturating amounts of NADPH.
Characterization of the Reductive Half Reaction

The reductive half reaction in 2-KPCC and all DSORs begin with the binding of NADPH and end with the generation of the reduced active site, which then binds and reduces substrate. The reductive half is rate limiting in DSORs and can be orders of magnitude slower than the oxidative half reaction. I monitored the reductive half reaction of 2-KPCC by stopped flow UV/Vis (15μM 2-KPCC, 150μM NADPH, pH 6, 25°C). This concentration of NADPH was chosen because it is a saturating amount, which is where the reaction will not become any faster with the addition of more NAPDH. Initially observed was a loss in absorptivity of the flavin peak at 460nm, corresponding to reduction. Then, the peak slowly grew back in intensity before finally and rapidly decaying into a final species with a spectrum resembling a 1e- photoreduced flavin. This is supported by control experiments in which the enzyme was pre-reduced by NADPH and then exposed to the spectrophotometer’s xenon lamp (i.e the full spectrum of visible light, white light), this experiment yielded identical final spectra (Figure 7).

What should be noted in the spectra is the lack of charge transfer band (540 nm); this band would arise from an interaction between the oxidized FAD and the charge transfer cysteine. This species is the reactive form of glutathione reductase and is stabilized by the charge on the active site histidine. However, 2-KPCC lacks the active site histidine which would potentially stabilize this charge transfer species; therefore, it is potentially unlikely that the charge transfer species would be the active form. This is supported by the lack of charge transfer band in the observed spectra.
In order to visualize the events of reaction more clearly and without the interference of photoreduction, the reductive half reaction was monitored again with UV/Vis excitation at a single wavelength (460nm) using the stopped flow UV/Vis in photomultiplier detection. By using the stopped flow UV/Vis in the photomultiplier detection mode, it does not monitor the reaction using the same harsh bright light that the diode array detection uses and instead uses monochromatic light. The monochromatic light will not cause photoreduction.

The Reductive Half Reaction using Single Turnover Wavelength Excitation and Photomultiplier Detection

The reductive half reaction in 2-KPCC is slow relative to typical DSORs and occurs in multiple observable phases. The transient reduction and re-oxidation of the flavin were most distinct at low pH. The initial two phases, which correspond to the reduction and re-oxidation of FAD were fit to single exponential curves which yielded $k = 27 \pm 2 \text{ s}^{-1}$ and $k = 10 \pm 2 \text{ s}^{-1}$, respectively (pH 6.5, 100 mM KPi, 200 mM NaCl, 150 µM NADPH). The third phase of reaction had not been previously seen because it had been masked by photoreduction, which the use of the monochromatic light avoids (Figure 8). This third phase present is still undefined but corresponds to changes in the chromophore as it reaches its final form. As the pH was raised, the distinct flavin reduction and re-oxidation steps eventually merged into one phase. The source of this effect is unclear at this time.
WT vs C82A Multi-Site Binding of NADP$^+$

In glutathione reductase the active form is the two-electron reduced state with NADP$^+$ bound and two protons in the active site (EH$_2$), with one proton on the active site histidine (HisH$^+$) and the other on Cys$_{\text{INT}}$. The charge on the HisH$^+$ stabilizes the charge transfer interaction between the oxidized FAD and the Cys$_{\text{CT}}$, and the NADP$^+$ is critical for stabilizing the C4a-adduct and catalysis. What should be noted is that the active site of glutathione reductase has multiple protonation states available, however, only the EH$_2$ and EH$^-$ forms experience activity, based upon pH (Figure 9a), and the EH$_2$ state is the most active. In this study the catalytically reactive, two-electron-reduced 2-KPCC-NADP$^+$ species is the same form whose protonation state and electronic structure are being explored here (Figure 9b). The pKa of Cys$_{\text{CT}}$ was previously measured in the absence of NADP$^+$ using the 2–KPCC Cys$_{\text{INT}}$ mutant (Cys82Ala = Cys$_{\text{INT}}$Ala), where there is only one titratable cysteine. However, with NADP$^+$ being crucial for catalysis, the UV/Vis spectrum of the Cys$_{\text{INT}}$Ala-NADP$^+$ complex was monitored as a function of pH by Dr. Greg Prussia.

In order to inform experiments done by Dr. Prussia it was necessary to determine the NADP$^+$ binding affinities for both the wild type and the C82A mutant (Cys82Ala = Cys$_{\text{INT}}$Ala). This was done using titrimetric addition of increasing quantities of NADP$^+$ to 2-KPCC which leads to a decrease in the enzyme’s maximal fluorescence signal at 525nm (excitation 460nm; Figure 10). What was found was that both wild type and C82A mutant proteins show multi-site binding of NADP$^+$ with high affinity, which reflects the homodimeric nature of 2-KPCC and is common in DSORs.
This informed Dr. Prussia’s experiments which determined that 2-KPCC does not form a C4a-adduct and that the charge transfer species is only formed at high pH (pKa = 9.4 ± 0.1). He monitored changes in the UV/Vis spectrum of the Cys\textsubscript{INT}Ala-NADP\textsuperscript{+} complex of 2-KPCC as a function of pH. This mutant only has one titratable active site cysteine and from this he determined the low pH form of 2-KPCC does not form the flavin-C4-Cys\textsubscript{CT} adduct,\textsuperscript{20} demonstrating that the reactive form of 2-KPCC is distinct from glutathione reductase. My measurements of the NADP\textsuperscript{+} K\textsubscript{D} informed him how much NADP\textsuperscript{+} he needed in order to ensure that it was bound to 2-KPCC while he did his measurements.

**Addition of an Active Site Proton Donor Residue in F501H**

Most DSOR members (such as glutathione reductase) have a catalytically essential histidine. This active site histidine is important for stabilizing the active form in glutathione reductase and acts as a proton source for forming glutathione (GSH). 2-KPCC contains a phenylalanine in the place of this histidine, which necessitates that the steps of reaction and active form are different than glutathione reductase. This is supported by the reductive half reaction proceeding significantly more slowly in 2-KPCC than in glutathione reductase with kinetics much more similar to the H439A mutant of glutathione reductase, as was discussed previously in chapter one, typical flavin and cysteine containing oxidoreductases (DSORs) compared to 2-KPCC\textsuperscript{12,13} (Figure 4). Furthermore, the slowing of the reaction in the H439A mutant of glutathione reductase allows for the observation of the flavin reduction and slow flavin reoxidation phases which are also observable in the 2-KPCC reaction. This further suggests that the lack of histidine allows for the reduction of the 2-
KPCC active site to undergo the reductive half reaction in different steps than glutathione reductase.

The reductive half reaction was monitored for the F501H mutant at low (pH 7, 100 mM KPi, 200 mM NaCl) and high (pH 9, 100 mM Glycine-NaOH, 200 mM NaCl) (Figure 11). At low pH the two observable phases correspond to the loss and gain of absorptivity by the flavin chromophore (flavin reduction and reoxidation), followed by photoreduction caused by the intense white light used during diode array detection. At high pH these phases converge into one slower phase, followed by photoreduction. The cause of this convergence caused by pH is not clear. The mutant and wild type were very similar with a moderate acceleration in rate constants for the F501H mutant. This leads us to believe that a histidine in this position does not substantially accelerate the rate of reduction as it does in glutathione reductase.

Summary of CO$_2$-fixing Flavoenzyme

Previous studies have shown that replacing phenylalanine with histidine makes the product distribution shift to acetone.$^{21}$ The hydrophobic phenylalanine at this position steers the reactive enolacetone toward carboxylation. The absence of an active site histidine in 2-KPCC guarantees that its protonation state is different from the reactive EH$_2$ species in glutathione reductase.

The data suggest two possible candidates for the reactive form. Either the EH$_2$ species forms with both Cys$_{\text{INT}}$ and Cys$_{\text{CT}}$ protonated, or an EH$^+$ species where a single proton is bound to Cys$_{\text{INT}}$ and shared in a close hydrogen bond interaction with Cys$_{\text{CT}}$. However, we reasoned that the EH$_2$ species is the least likely of the two. Prior work
studying DSORs suggests $\text{Cys}_{\text{INT}}$ has to be deprotonated to be an effective nucleophile for breaking an S-S bond$^{22-24}$. So we expect an even stronger nucleophile to be needed in order to break a stronger C-S bond. With the active site of 2-KPCC being dominated by F501, the hydrophobic environment does not support the easy proton loss by $\text{Cys}_{\text{INT}}$ in the $\text{EH}_2$ form. Therefore it is unlikely that $\text{Cys}_{\text{INT}}$ it would be the effective nucleophile needed in order to break the C-S bond of 2-KPC, in the $\text{EH}_2$ form.

Alternatively, such a shared proton interaction has been proposed for the cysteine disulfide of thioredoxin reductase$^{15,25}$ and a variety of other enzymes without reactive disulfide. Sharing a proton between the two sulfur atoms in thioredoxin reductase stabilizes a reactive thiolate anion at neutral pH, and a stable thiolate is essential for reduction of substrate$^{15}$.

To determine the protonation state of reduced 2-KPCC three sets of experiments were carried out by Dr. Bennett Streit, all of which were published in Streit et al 2019. First, bromoethanesulfonate (BES) a suicide inhibitor which cross-links irreversibly with the $\text{Cys}_{\text{INT}}$$^{26,27}$ was incubated with pre-reduced enzyme at varying pH values. The C-Br group in BES serves as a surrogate for the C-S bond in 2-KPC and Br$^-$ acts as the leaving group. Only deprotonated $\text{Cys}_{\text{INT}}$ is expected to cross-link with BES$^{26,27}$. Therefore, the pH dependence of 2-KPCC inactivation is a measure of the $\text{Cys}_{\text{INT}}$-SH pKa. The data showed that formation of an irreversible cross-link increases at elevated pH, with a pKa = 8.1 ± 0.1, which we attribute to the $\text{Cys}_{\text{INT}}$-SH.

Second, the steady state kinetics of the 2-KPCC catalyzed reaction between NADPH and 2-KPC in the presence and absence of CO$_2$ were monitored as a function of
[2-KPC] and of pH. Michaelis plots of initial rate versus [2-KPC] show substrate inhibition at relatively high concentrations of 2-KPC and a dependence on pH. The parameter $k_{cat}/K_M$ [2-KPC], reports on all reactions steps involving 2-KPC up to and involving the rate limiting step. The plot of this parameter versus pH is bell shaped, peaking at 8.5 and with pKa near 7.5. The increase in reactivity is attributed to the pKa for Cys$\text{INT}$, with a decrease in $k_{cat}/K_M$ [2-KPC] above this pH due to enzyme inactivity. The two pH-rate profiles in this experiment share a similar pKa to the one determined for Cys$\text{INT}$ in the BES-inactivation experiment described previously, which again is attributed to the Cys$\text{INT}$ proton. Observations of pKas shifted downward from expected values are common in systems with sidechain-shared protons.$^{28}$ A pKa of 7.5-8.1 for Cys$\text{INT}$ in the absence of any base or negative charge stabilizing group implies the involvement of Cys$\text{CT}$ in a shared hydrogen bonding interaction.

Lastly, a proton inventory experiment was carried out. In a proton inventory experiment, the rate of reaction is monitored in a series of buffers with varied amounts of deuterated versus protonated water. The dependency of the rate on the mole fraction of $D_2O/(D_2O + H_2O)$ provides a readout of how many protons shift position in the active site in a reaction.$^{29}$ We monitored the rate of reaction at which BES formed a chemical cross-link with reduced 2-KPCC as the fraction of deuterium was increased. The data showed complete independence of the rate of this reaction step in the presence of deuterium. Therefore this experiment showed that no protons exchanged during the cross-linking reactions with BES (Figure 12).
Conclusions

2-KPCC is unique in the DSOR family because it uses a reduced disulfide to cleave a strong C-S bond, which then leads to the generation of an enolacetone intermediate that can directly attack and fix CO₂. This occurs while protecting the enolacetone from H⁺ which will lead to the unwanted product, acetone. It accomplishes this by having a unique reactive form that is distinct from better studied DSORs (such as glutathione reductase). The data suggests the reduced reactive form of 2-KPCC is characterized by the sharing of a single proton between Cys₇ and Cys₁₀. This species lowers the pKa of the Cys₁₀ in this strongly hydrophobic environment to be between 7.5-8.1. Also, Cys₇ can readily assume ownership of the proton while Cys₁₀ nucleophilically attacks the substrate, thereby protecting the enolacetone intermediate from free protons.
Figure 1. Relative speciation and reactions of activation of carbon dioxide. (A) Relative speciation (%) of carbon dioxide, bicarbonate, and carbonate in water as a function of pH, figure found in Pederson et al 2013. (B) Reaction of bicarbonate with phosphoenolpyruvate to make a carboxyphosphate intermediate. (C) Reaction of bicarbonate with ATP and then the phosphorylated intermediate reacting with biotin to create the carboxybiotin intermediate. (D) A basic carboxylation reaction showing a nucleophilic carbon attacking CO$_2$ to generate a new C-C bond. Figure inspired by Frey et al 2007.
Figure 2. Overview of RuBisCO reactions. The substrate of RuBisCO, ribulose-1,5-bisphosphate (RuBP) undergoing enolization to make an intermediate which can react with either oxygen or carbon dioxide. In the oxygenation reaction the end products are 2-phosphoglycolate and 3-phosphoglycerate. Where 2-phosphoglycolate will lead into the photorespiration pathway where it can be salvaged and made into 3-phosphoglycerate. 3-phosphoglycerate will continue in the Calvin cycle and eventually be made into energy for the cell. The carboxylation step makes two molecules of 3-phosphoglycerate where it can bypass the salvage pathway of photorespiration and enter the Calvin cycle immediately. Figure inspired by Anderson et al 2008.
Figure 3. Overview of representative carboxylases. (A) Overview of RuBisCO reaction, showing substrates RuBP and CO₂ converting to products, glycerate-3-phosphate. (B) Overview of PEP-C reaction showing phosphoenolpyruvate converting to oxaloacetate. (C) Overview of Acetyl-CoA carboxylase (ACC) showing acetyl CoA converting to malonyl CoA. Inspired by Erb et al 2011
Figure 4. Canonical steps by which the active site of DSORs are reduced, yielding the reactive form of the active site. The oxidized form of the enzyme (1) rapidly binds NADPH (2). A hydride is transferred from NADPH to the flavin (3), rendering its characteristic yellow chromophore colorless. The flavin subsequently transfers two electrons to the nearby disulfide in a process catalyzed by the active site histidine, yielding a C4a-flavin-CysCT covalent intermediate (4). The covalent species rapidly tautomerizes to yield the catalytically active CT complex (6), which is stabilized by the active site HisH+. Stopped flow kinetics studies of the reductive half reaction of glutathione reductase identified two phases, diagrammed by the arrows. In the first phase, the oxidized flavin is converted to the reduced form (3). In the second, slightly slower phase, the flavin reoxidizes to yield the reactive CT species (5). Second order rate constants for each phase are given for the WT GR. Values for the active site histidine mutant (H439A) are given in parentheses, found in Rietveld et al 1994. Notably, formation of the reduced flavin is only clearly observed for the H439A mutant, due to >10-fold slowing of the reoxidation of the flavin. Rate constants measured for the analogous two phases in 2-KPCC were orders of magnitude smaller: $k = 27 \pm 3 \text{ s}^{-1}$ and $10 \pm 2 \text{ s}^{-1}$ (pH 6.5, 25 °C) respectively. Figure from Streit et al 2019.
Figure 5. Proposed oxidative half reaction for 2-KPCC under carboxylating conditions. Note that the enolacetone intermediate created in the first step of the 2-KPCC reaction can react with any available protons to form the off-pathway product acetone. Restricting water or other sources of protons from the active site is therefore critical for reaction fidelity. At the same time, the carboxylation reaction as proposed does not require the entry or loss of protons from the active site, and instead uses Cys\textsubscript{CT} as an internal acid-base.
Figure 6. The rate of 2-KPCC reduction by NADPH saturates at low concentrations. The reductive half reaction between 2-KPCC (15 μM) and NADPH was monitored at 460 nm by stopped-flow spectrometry using 2 (black), 10 (blue), 15 (red), or 20 (green) equivalents of NADPH. Each curve could be fit to the sum of three exponential functions (solid black lines), reflecting three consecutive kinetic events. (Note that since single wavelength excitation was used, there is no photoreduction phase at the end of the reaction.) The initial phase (< 0.1 s) is associated with large changes at 460 nm, indicative of an initial flavin reduction. (Inset) The rate constant for the initial kinetic phase is plotted as a function [NADPH]. The rate constant reaches a saturating value by 150 μM NADPH (10 eq). Figure found in Streit et al 2019.
Figure 7. The slow reductive half reaction of 2-KPCC has flavin reduction and reoxidation steps followed by photoreduction. The reaction between anaerobic 15 μM 2KPCC (red trace) and 10 eq of NADPH at pH 6 (100 mM KPi) results in large changes to the flavin spectrum over time. After 100 ms (blue) and by 1s (orange trace) the intensity of the flavin absorbance decreases by ~2-fold. This is followed by an increase and slight blue shift in the flavin absorbance, maximizing at 70 s (green). Under the bright light used for diode array detection, photoreduction of the oxidized flavin, accompanied by increases in absorbance at 550-700 nm, occurs subsequently over longer time scales, indicative of formation of a flavin semiquinone (200 and 700 s; purple and black traces). Figure found in Streit et al 2019.
Figure 8. The reductive half reaction in 2-KPCC is slow relative to typical DSORs and occurs in multiple observable phases. (A) The reaction of 15 μM 2-KPCC at pH 6.5 (100 mM phosphate buffer) with 10 eq of NADPH monitored at 460 nm by stopped flow exhibited three kinetic phases. The first two phases correspond to loss and gain of absorptivity by the flavin chromophore (flavin reduction and reoxidation) and were fit to single exponential curves to yield $k = 27 \pm 3 \text{ s}^{-1}$ and $k = 10 \pm 2 \text{ s}^{-1}$ (errors are ± 1 standard deviation from 3 measurements). The third phase corresponds to subsequent, still undefined changes in the chromophore as it reaches its final form. Note that the use of monochromatic light avoids the photoreduction phase, which obscures the final phase when white light excitation is used (Figure 5). (B) Reaction of 15 μM 2-KPCC at varying pH (6.5-9.5) with 10 eq of NADPH was monitored at 460 nm by stopped flow. At pH 6.5 (red), 7 (orange), and 7.5 (gold) three kinetic phases are observed. At pH 8 (green), 8.5 (light blue), 9 (dark blue), and 9.5 (purple) the two phases have converged. Figure found in Streit et al 2019.
Figure 9. Possible protonation states for the glutathione reductase and 2-KPCC active sites. (A) All of the possible protonation states for glutathione reductase, also showing the active form, EH$_2$. Inspired by Williams et al 1994. (B) Protonation states of 2-KPCC. The reduced, reactive form of 2-KPCC at neutral pH could be either an EH$_2$ or EH$^-$ form. The EH$_2^-$ form is considered unlikely to be biologically relevant because $pK_a 2$ is potentially too high for a substantial amount of this species to accrue. Moreover, because the spectrum for reduced 2-KPCC exhibits no CT band, only the non-CT species are possible candidates, and we therefore rule out both species EH$_2^-$ and EH$^-$ with the charge transfer, leaving EH$_2$ and EH$^-$ without the charge transfer interaction, as possible models for the active site. The EH$^-$ form in (C) stabilizes a nucleophilic thiolate, similar to the proposed reactive form for thioredoxin active sites (Cheng et al 2011) Note: the canonical reactive form for DSORs resembles form EH$^-$ with the charge transfer band, but with HisH$^+$ available to stabilize the flavin-CysCT charge transfer interaction. Since both the histidine and Cys$_{INT}$ bear protons, it is an EH$_2$ state. Figure inspired by Streit et al 2019.
Figure 10. Wild type and C82A (C82 = CYSINT) 2-KPCC show multisite binding of NADP\(^+\) with high affinity. Addition of NADP\(^+\) to 2-KPCC leads to a decrease in the enzyme’s maximal fluorescence signal at 525 nm (460 nm excitation). A) The change in fluorescence at 525 nm was plotted as a function of added NADP\(^+\) concentration (100 mM potassium phosphate, pH 6.5). The data were fit to the sum of two Langmuir isotherms, yielding \(K_{D1} = 3.8\ \mu\text{M}\) and \(K_{D2} = 3.2\ \mu\text{M}\). The identification of two slightly different values for \(K_D\) reflects the homodimeric nature of 2-KPCC and is common among DSORs. B) The titration was repeated at pH 9.5 (100 mM CAPS). The data were again fit to the sum of two Langmuir isotherms, yielding \(K_{D1} = 4.6\ \mu\text{M}\) and \(K_{D2} = 4.2\ \mu\text{M}\). C) The titration was repeated with the C82A mutant (5 mM 2-KPCC C82A, 100 mM CAPS, pH 9.5) and was plotted as a function of added NADP\(^+\) concentration. The data were fit to the sum of two Langmuir isotherms, yielding \(K_{D1} = 17\ \mu\text{M}\) and \(K_{D2} = 20\ \mu\text{M}\). Figure found in Streit et al 2019.
Figure 11. Addition of an active site proton donor residue in F501H has no impact on the rate of 2-KPCC reduction by NADPH. Reactions between WT or F501H 2-KPCC (15 μM) at either pH 7 (100 mM phosphate) or 9.0 (100 mM Tris-Cl) were monitored to examine the effect of substituting histidine into the 2-KPCC active site at position 501 in the active site. The initial phase of the reactions at pH 7.0 (Red – Bold line (WT) and fine line (F501H)) and pH 9.0 (Blue – bold line (WT) and fine line (F501H)) are largely similar at their respective pH values, with only modest accelerations in the rate constants for the F501H mutant. We conclude that a histidine in this position does not substantially accelerate the rates of active site reduction as in other members of the DSOR protein family, where rates of the initial flavin reduction phase are ~10 x higher than 2-KPCC. Figure found in Streit et al 2019.
Figure 12. Prerduced 2-KPCC forming an irreversible crosslink with suicide inhibitor BES. This shows the proposed reduced, catalytically active form of 2-KPCC’s active site reacting with the suicide inhibitor BES. Figure inspired by Boyd et al 2010.
REFERENCES CITED


24. Jursic, B. S. Computation of Bond Dissociation Energy for Sulfides and Disulfides


APPENDIX

Manuscript Information

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CHAPTER APPENDIX

THE REACTIVE FORM OF A C-S BOND-CLEAVING CO$_2$ FLAVOENZYME

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The reactive form of a C-S bond-cleaving, CO$_2$-fixing flavoenzyme

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ABSTRACT

NADPH:2-ketopropyl-coenzyme M oxidoreductase/carboxylase (2-KPCC) is a bacterial disulfide oxidoreductase (DSOR) that, uniquely in this family, catalyzes CO$_2$ fixation. 2-KPCC differs from other DSORs by having a phenylalanine that replaces a conserved histidine, which in typical DSORs is essential for stabilizing the reduced, reactive form of the active site. Here, using site-directed mutagenesis and stopped-flow kinetics, we examined the reactive form of 2-KPCC and its single turnover reactions with a suicide substrate and CO$_2$. The reductive half reaction of 2-KPCC was kinetically and spectroscopically similar to that of a typical DSOR, glutathione reductase, in which the active-site histidine had been replaced with an alanine. However, the reduced, reactive form of 2-KPCC was distinct from those typical DSORs. In the absence of the histidine, the flavin and disulfide moieties were no longer coupled via a covalent or charge transfer interaction as in typical DSORs. Similar to thioredoxins, the pK$_a$ between 7.5 and 8.1 that controls reactivity appeared to be due to a single proton shared between the cysteines of the dithiol, which effectively stabilizes the attacking cysteine sulfide and renders it capable of breaking the strong C-S bond of the substrate. The lack of a histidine protected 2-KPCC’s reactive intermediate from unwanted protonation;
however, without its input as a catalytic acid-base, the oxidative half reaction where carboxylation takes place was remarkably slow, limiting the overall reaction rate. We conclude that stringent regulation of protons in the DSOR active site supports C-S bond cleavage and selectivity for CO₂ fixation.

Atmospheric CO₂ serves as the carbon source for building the biomass of photosynthetic plants and chemoautotrophic microbes. In phototrophs, the first step of CO₂ fixation is catalyzed by ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), the most abundant enzyme on earth (1). A variety of processes that fix CO₂ or its hydrated form (bicarbonate, HCO₃⁻) in non-photosynthetic microbes and eukaryotes, most often using biotin to transport CO₂, have likewise been described (2). How these processes contribute to the global carbon cycle, and the catalytic paradigms by which many of the relevant enzymes capture and transform this important greenhouse gas, are not fully understood.

NADPH:2-ketopropyl-coenzyme M oxidoreductase/carboxylase (2-KPCC) is a bacterial enzyme that catalyzes the direct fixation of CO₂ into biomass as part of a pathway for metabolizing small alkenes, including propylene gas (3). This enzyme belongs to a group of flavin adenine dinucleotide (FAD) and cysteine-disulfide containing oxidoreductases (DSORs) that are best known for reducing disulfide (glutathione or lipoamide reductase) or metal ion (mercuric reductase) substrates.

DSORs share a common mechanism that can be divided into two halves (4). In the slower reductive half (Figure 1), the hydride of NADPH is transferred via the FAD cofactor to a conserved cysteine disulfide. The reduced active site accumulates in a reactive, doubly protonated form (EH₂) where the cysteine proximal to the FAD (known as the charge-transfer thiol, Cys₇₅SH) participates in a charge-transfer interaction with the oxidized flavin (Cys₇₅S···FAD). This CT species is stabilized by the charge on the active site acid-base histidine (Figure 2, Figure 3C). (4) The cysteine distal to the FAD, known as the interchange thiol (CysᵥSH), has its pKₐ lowered to 7.5 by the proximity of the active site HisH⁺, and is thereby poised to reduce a substrate in the enzyme’s oxidative half reaction. In glutathione reductase (GR), a well characterized model DSOR, glutathione disulfide (GSSG) is reductively cleaved by CysᵥSH and protonated by HisH⁺ to form one molecule of glutathione (GSH) and a covalent, mixed disulfide intermediate (CysᵥS-GSH). Protonation of the covalent intermediate then yields the second equivalent of GSH and allows the oxidized cysteine disulfide (Cys₇₅S-SᵥCys) to reform.

The oxidoreductase platform of DSORs is uniquely repurposed by 2-KPCC for catalyzing a carboxylation reaction following reductive bond cleavage. Instead of a disulfide substrate, 2-KPCC reductively cleaves the relatively strong C-S bond of a thioether substrate known as 2-KPC (2-ketopropyl-coenzyme M, or [2-(2-ketopropylthio)ethanesulfonate]) (5,6). The initial cleavage product is an unstable enolacetone anion which nucleophilically attacks enzyme-bound CO₂ to form the new carbon-carbon bond of acetoacetate (Figure 4). In the absence of CO₂ and in
the presence of available protons, the protonation product acetone forms in an unwanted side reaction.

Structurally, 2-KPCC lacks the catalytically important, conserved histidine that is shared by most other members of its family. Substitution of the histidine by alanine leads to loss of nearly all catalytic function in GR (4,7); however, the native residue at this position in 2-KPCC is a phenylalanine (Phe501) (Figure 3D).

We previously showed that substitution of a histidine at this position (Phe501His) completely shifted the product outcome from acetoacetate (carboxylation product) to acetone (protonation product) (8). The hydrophobic residue at this position in 2-KPCC seemingly steers the reactive enolacetone intermediate toward carboxylation and away from protonation. However, because 2-KPCC lacks the catalytic input of the histidine, we hypothesized that both reaction halves might proceed differently than in typical DSORs. Moreover, the reduced form of the active site must likewise be distinct and, because it lacks the conserved active site histidine, must necessarily have a unique protonation state (9). We therefore sought to characterize the reduced, reactive form of 2-KPCC and its reaction with substrates and substrate analogs, to determine how it cleaves a strong thioether bond while avoiding production of acetone (5,6). Our results suggest that 2-KPCC has a novel reactive electronic and protonation state among DSORs. We propose a mechanism in which, unlike canonical DSORs, the cysteine proximal to the FAD acts as an internal acid-base, requiring no additional protons and strongly favoring carboxylation over the competing protonation pathway.

RESULTS

The reductive half reaction in WT 2-KPCC is biphasic and slow. The 2-KPCC reaction can be divided into reductive and oxidative halves. The reductive half reaction in 2-KPCC and all DSORs starts with NADPH binding and ends with the generation of the two-electron reduced active site (Figure 1) (4,10). This is the catalytically competent form of the enzyme which binds to and reduces substrate. The reductive half is rate limiting in DSORs, and may be orders of magnitude slower than the second, oxidative half, in which the active site reduces the exogenous substrate.(11)

We monitored the reductive half reaction of 2-KPCC (Figure 3) by stopped flow UV/vis under conditions where NADPH was saturating (15 μM enzyme, 150 μM NADPH, pH 6, 25 °C) (Figure S1). A clear loss in absorptivity was initially observed in the characteristic flavin peak at 460 nm, corresponding to transient flavin reduction (Figure 5). The peak then slowly grew back in intensity, before rapidly decaying into a final species with a spectrum resembling a 1e- photoreduced flavin (14). Control experiments in which the enzyme was first reduced by NADPH and then exposed to the spectrophotometer’s xenon lamp yielded identical final spectra, supporting this interpretation (Figure S2).

The transient reduction and reoxidation of the flavin were most distinct at low pH. In order to visualize each of these events clearly and without interference from the final photoreductive phase, the reductive half reaction was monitored again with UV/vis absorption at a single wavelength (460 nm) at pH 6.5 (Figure 6A). The initial two phases corresponding to the reduction/reoxidation of FAD were fit to
single exponential curves to yield $k = 25 \pm 2$ and $k = 13 \pm 2 \text{ s}^{-1}$, respectively. Both rate constants are orders of magnitude smaller than for the analogous reductive half reaction of GR (4), and are much closer to the rate constants measured for the His439Ala mutant of GR (Figure 1). We conclude that the presence of a hydrophobic residue at this position (GR His439Ala or (2-KPCC Phe501) at this position has a broadly similar effect (Figure 6A), which is to dramatically slow both the reduction and reoxidation of the flavin (4,7). As the pH was raised, the distinct flavin reduction and reoxidation steps eventually merged (Figure 6B) (4). The source of the pH effect is not clear.

The catalytically active, reduced form of 2-KPCC is neither a CT nor a C4a-adduct species. We next sought to characterize the electronic structure and protonation state of the reduced form of 2-KPCC, and compare it with typical DSORs. This is the form of the protein that has previously been shown to bind to and reduce exogenous substrates in DSORs as well as in 2-KPCC (12). The most extensive studies have been carried out using GR, showing that it reacts with glutathione in its two-electron reduced state with NADP$^+$ bound. This form (designated as EH$_2$) has two protons in the active site. One proton resides on the conserved histidine (HisH$^+$) and the other on Cys$_{\text{INT}}$ (4). The charge on HisH$^+$ stabilizes a charge transfer interaction between the flavin and Cys$_{\text{CT}}$, and lowers the pKa on Cys$_{\text{INT}}$ to near 7.5 (10). This primes Cys$_{\text{INT}}$ to lose a proton prior to nucleophilic attack on glutathione (Figure 2). Below pH 7.5, the EH$_3^+$ form predominates, in which the Cys$_{\text{CT}}$ sulfide forms a covalent bond with the flavin C4a carbon. The three protons in this case are located on the flavin nitrogen, the active site histidine, and Cys$_{\text{INT}}$. The EH$_3^+$ form is largely unreactive.

The two-electron-reduced 2-KPCC-NADP$^+$ complex is, by analogy, the catalytically reactive form of 2-KPCC whose protonation state and electronic structure were studied here. The pKa of Cys$_{\text{CT}}$ was previously measured in the absence of NADP$^+$ using the 2-KPCC Cys$_{\text{INT}}$ variant (Cys82Ala or Cys$_{\text{INT}}$Ala), which has just one titratable active site cysteine. A pKa >9.0 was determined, reflecting the hydrophobic environment of this active site (Figure 3) (17). NADP$^+$ is critical both for stabilizing the C4a–adduct and for catalysis (16). We therefore monitored changes in the UV/vis spectrum of the Cys$_{\text{INT}}$Ala-NADP$^+$ complex of 2-KPCC as a function of pH. (See Figure S3 for determination of NADP$^+$ binding affinities.) Remarkably, the acidic form of 2-KPCC did not possess the characteristic spectrum of a flavin-C4a-Cys$_{\text{CT}}$ adduct (16). Moreover, the charge transfer species only formed at high pH (pKa = 9.4 ± 0.1), in spite of the presence of the nearby positively charged nicotinamide ring (Figure 7). Hence, distinct from canonical DSORs, the reactive form of 2-KPCC at neutral pH is neither a charge transfer species nor a C4a-adduct but, rather, a species with a unique electronic structure.

The reactive form of 2-KPCC may be a nucleophilic EH-species. The lack of an active site histidine (Figure 2) in the 2-KPCC active site ensures that its protonation state must also be distinct from the reactive EH$_2$ species of GR. The structure of the 2-KPCC active site (Figure 3) and the available data suggest two possible descriptions for the reactive form (4) (Figure 2). The first is an EH$_2$ species in which both Cys$_{\text{INT}}$ and Cys$_{\text{CT}}$ are protonated. However, the immediate
environment around Cys\textsubscript{INT}, dominated by F501, is highly hydrophobic and devoid of basic residues. Such an environment is not expected to support facile proton loss by Cys\textsubscript{INT}. Prior work with DSORs suggests Cys\textsubscript{INT} must be deprotonated to be an effective nucleophile for breaking S-S bonds (9). Hence, we expect Cys\textsubscript{INT} deprotonation to precede cleavage of the stronger C-S bond of 2-KPC.

Alternatively, the active form could be an EH- species, in which a single proton is bound to Cys\textsubscript{INT} and shared in a close hydrogen bonding interaction with Cys\textsubscript{CT}. Importantly, such a shared-proton interaction has been proposed for the cysteine disulfide of thioredoxins (9), as well as other diverse enzymes without reactive disulfides, including aspartic proteases (18,19), myoglobin (20), bacteriorhodopsin (20,21), and ribonuclease H1 (22). Sharing the proton between the two sulfur atoms in thioredoxins (enzymes which do not possess an accompanying flavin) effectively stabilizes a reactive thiolate anion at neutral pH (9). As in DSORs, a stable thiolate is deemed essential for reduction of substrate.

To assess the reactive protonation state of reduced 2-KPCC, three sets of experiments were carried out. First, 2-KPCC (15 $\mu$M) was anaerobically pre-reduced with DTT and then incubated anaerobically with excess (10 mM) bromoethanesulfonate (BES) at varying pH values. Upon incubation of BES with DTT reduced enzyme it was previously shown that BES slowly and irreversibly cross-links with Cys\textsubscript{INT} in 2-KPCC as well as in classic DSORs. The C-Br bond in BES is a surrogate for the C-S bond in 2-KPC, and Br\textsuperscript{-} is the leaving group (23). Only deprotonated Cys\textsubscript{INT} is expected to cross link with BES (23); hence, the pH dependence of 2-KPCC inactivation serves as a functional measure of the Cys\textsubscript{INT}-SH pKa. The data in Figure 8 showed that formation of an irreversible cross link increases at elevated pH with a pKa = 8.1 $\pm$ 0.1, which we attribute to the Cys\textsubscript{INT}-SH.

Second, the steady state kinetics of the 2-KPCC-catalyzed reaction between NADPH and 2-KPC in the presence and absence of CO\textsubscript{2} were monitored as a function of [2-KPC] and at varying pH values. When CO\textsubscript{2} is absent, the enolacetone intermediate produced from 2-KPC cleavage reacts with H\textsuperscript{+} to form acetone. Note that varying the pH has the effect of modulating both the protonation state of Cys\textsubscript{INT} and the concentration of protons available for reaction with the enolacetone, from 0.32 $\mu$M (pH 6.5) to 0.32 nM (pH 9.5). Michaelis plots of initial rate versus [2-KPC] demonstrated substrate inhibition at relatively high concentrations of 2-KPC (Figure S4), and a clear dependence on pH. The parameter $k_{cat}/K_M$[2-KPC], which reports on all reaction steps involving 2-KPC up to and including the one that is rate limiting, is plotted versus pH in Figure 9. The plot is approximately bell shaped, peaking at pH 8.5 and with a pKa near 7.5. We interpret the increase in reactivity to the pKa for Cys\textsubscript{INT} (Figure 9). The decrease in $k_{cat}/K_M$[2-KPC] above this pH is most likely due to enzyme inactivation.

The enolacetone preferentially reacts with CO\textsubscript{2} when it is saturating to form acetoacetate (8), as shown in prior work. The parameter $k_{cat}/K_M$[2-KPC] under CO\textsubscript{2}-saturated conditions has the same bell shape for its pH-rate profile, and the same pKas as in the absence of CO\textsubscript{2}. This suggests that pH has the same influence on steps up to and preceding the rate limiting
step of the reaction with 2-KPC, regardless of whether the enolacetone intermediate reacts with $H^+$ or $CO_2$.

The two pH-rate profiles in Figure 9 share the pKa determined for $Cys_{INT}$ in the BES-inactivation experiments (Figure 8), which we again tentatively ascribe to the $Cys_{INT}$ proton. A hallmark of systems with sidechain-shared protons is the observation of pKas shifted downwards from expected values (9). For a cysteine buried in a hydrophobic environment (25), we expect the pKa to be above the free-cysteine value of 8.5. Indeed, the pKa for $Cys_{CT}$ in the $Cys_{INT}Ala$ mutant is elevated to above 9.4. The pKa of 7.5 for $Cys_{INT}$, despite the absence of any general base or negative charge-stabilizing group, suggests the involvement of $Cys_{CT}$ in a shared hydrogen bonding interaction.

Notably, the values of $k_{cat}/K_M[2-KPC]$ are 1-2 orders of magnitude larger for the carboxylation of enolacetone than for its protonation, across the pH range. The reasons for faster steady state turnover with $CO_2$ are not clear. However, we note that the concentration of $CO_2$ under our experimental conditions, where $CO_2$ is introduced by adding a large excess of KHCO$_3$ (60 mM) to each buffer solution, is both kinetically saturating for the steady state reaction (data not shown) and likely orders of magnitude higher than the corresponding concentration of $H^+$ across the pH range used ([H$^+$] = 320 – 0.32 nM as the pH varies from 6.5 – 9.5). We postulate that the faster observed turnover with $CO_2$ is due to the relatively higher concentration of $CO_2$ versus $H^+$.

As a third and final test of the EH-hypothesis, a proton inventory experiment was carried out. In these experiments, the rate of a reaction is monitored in a series of buffers with varied amounts of $^2H_2O$ ($D_2O$) versus H$_2$O. The dependency of the rate on the mole fraction of $D_2O/(D_2O + H_2O)$ provides a readout on how many protons shift positions in the active site in the reaction or chemical step under study (26). Here, we monitored the rate at which BES formed an irreversible cross-link with the reduced form of 2-KPCC as the fraction of deuterium in the buffer was increased (Figure 10). The data exhibited complete independence of the rate of this step on the heavy isotope. This experiment therefore showed conclusively that no protons exchange places in the reduced 2-KPCC active site as $Cys_{INT}$ cross-links with BES. This result strongly supports a model in which $Cys_{CT}$ and $Cys_{INT}$ share a single proton in the reduced, reactive form of 2-KPCC, permitting $Cys_{CT}$ to readily assume ownership of the proton as $Cys_{INT}$ nucleophilically attacks a substrate.

*The oxidative half reaction is slow relative to DSORs, but faster in the presence of excess CO$_2$.** In light of the predicted, more nucleophilic reduced active site of 2-KPCC, relative to typical DSORs, we wondered whether the oxidative half reaction with 2-KPC (Figure 4) might proceed rapidly. 2-KPCC was therefore reduced with a slight stoichiometric excess of NADPH, and the single turnover reaction with varying concentrations of 2-KPC was monitored over time, both in the presence and absence of saturating $CO_2$. In every case and similar to DSORs (4), the oxidative half reaction proceeded in a single kinetic phase, followed by photoreduction of the flavin. The data were fit to the sum of two exponential curves, where the first fitted rate constant encompassed the entire oxidative half reaction. The rate constants for the initial phase were plotted versus [2-KPC] and fit to a linear equation (Figure
yielding rate constants $k = 6.7 \times 10^{-4}$ μM s$^{-1}$ (CO$_2$ present) and $k = 1.1 \times 10^{-4}$ μM s$^{-1}$ (CO$_2$ absent). As with $k_{cat}/K_M$ of 2-KPC, the presence of CO$_2$ appeared to increase the rate of the oxidative half reaction, though again, the concentration of CO$_2$ is expected to be far higher than the concentration of H$^+$ under the conditions used.

At the highest concentrations of 2-KPC used here, the first order rate constants for the oxidative half reaction were $k = 0.14$ s$^{-1}$ (CO$_2$ present) and $k = 0.022$ s$^{-1}$ (CO$_2$ absent). By comparison, the analogous rate constant for the GR oxidative half reaction was many orders of magnitude faster: 3900 s$^{-1}$ in saturating glutathione (25 °C) (4). The reductive half reaction of 2-KPC was also relatively faster (rate constants $k = 27 \pm 3$ s$^{-1}$ and $k = 10 \pm 2$ s$^{-1}$ for the two phases). We conclude that, in contrast to typical DSORs, the oxidative half reaction limits the overall rate of catalysis for 2-KPC.

**DISCUSSION**

2-KPC is a member of a large class of oxidoreductases that contain FAD and a cysteine-disulfide. Unique among this family, 2-KPC uses the reduced disulfide to cleave a relatively strong C-S bond. This leads to generation of a reactive enolacetone intermediate that can directly attack and thereby fix CO$_2$ into biomass (Figure 4F). At the same time, 2-KPC effectively prevents H$^+$ from reaching the enolacetone intermediate and forming unwanted acetone. We hypothesized that the hydrophobic active site of 2-KPC, which lacks a conserved histidine (Figure 3), may be important for both C-S bond cleavage and carboxylation fidelity.

Our results here suggest that the hydrophobic active site of 2-KPC disfavors forming the flavin-Cys$^{CT}$ charge transfer intermediate, though this is the reactive form of the active site in all other well-studied DSORs (4, 27-30). In these enzymes, the active site Cys$^{INT}$ is rendered acidic (pKa ~ 7.5) by the nearby HisH$^+$ charge, making it a better nucleophile for attacking and reducing an exogenous substrate (4). The presence of an available proton so close to 2-KPCC’s enolacetone intermediate (Figure 4), however, appears to favor formation of the protonated rather than the carboxylated product, according to prior work with the Phe501His mutant (8).

This leads to a conundrum: how is 2-KPCC able to cleave the C-S bond of 2-KPC, without the activation that the active site HisH$^+$ provides to Cys$^{INT}$? We expect that a relatively stronger nucleophile should be required for this reaction than for S-S bond cleavage. The C-S bond of MeS-Me, for example, has a bond dissociation enthalpy that is ~20 kcal/mol higher than the S-S bond of MeS-SMe (5, 31). The potentially high pKa of cysteine in a hydrophobic environment would seem incompatible with the demands of the 2-KPCC reaction. Indeed, the pKa of Cys$^{CT}$ in the Cys$^{INT}$Ala variant is > 9.4.

We propose here that the remarkably low pKa observed for Cys$^{INT}$, which we estimate is between 7.5-8.1 (Figures 5-6) in spite of its hydrophobic environment, is due to proton sharing between Cys$^{INT}$ and Cys$^{CT}$ in an EH$^+$ complex (Figure 2) (9). This model is supported by proton inventory experiments, and it helps explain how Cys$^{INT}$ might be sufficiently activated to reductively cleave the C-S bond in 2-KPC, even in the absence of an active site HisH$^+$. Rather than forming a charge transfer interaction with the flavin, the Cys$^{CT}$ in 2-KPCC is instead poised to direct its electron density toward Cys$^{INT}$, effectively stabilizing a reactive Cys$^{INT}$
thiolate a hydrophobic environment that also stabilizes bound CO$_2$. This model is reminiscent of the shared-proton model proposed for thioredoxins (9), another family of enzymes that react via a cysteine disulfide. In those enzymes as well, a reactive thiolate is needed, often in a relatively hydrophobic environment.

Enclosing the 2-KPCC active site in a hydrophobic compartment that lacks an active site histidine is essential for promoting carboxylation instead of protonation of the enolacetone intermediate (8). By invoking a shared proton between Cys$^{CT}$ and Cys$^{INT}$ in reduced 2-KPCC, we can now propose a mechanism for the oxidative half reaction in which the acid-base functions of the active site histidine are performed instead by Cys$^{CT}$ (Figure 4). This likely renders the oxidative half reaction of 2-KPCC much slower than the equivalent half reaction in a canonical DSOR (4) (Figure 11), where delivery of protons from HisH$^+$ in GR, for example, is essential for rapid turnover (9). In fact, in 2-KPCC, the oxidative half reaction limits the overall reaction rate.

CONCLUSIONS
2-KPCC must balance the requirement for a strongly nucleophilic thiolate that is poised for C-S bond cleavage with the need to exclude reactive protons from the active site. We propose that it does so via proton sharing between Cys$^{CT}$ and Cys$^{INT}$. This lowers the pKa of Cys$^{INT}$ in a strongly hydrophobic environment. Cys$^{CT}$ then acts as a built-in, internal base to retain the proton once Cys$^{INT}$ reacts with 2-KPC, again protecting the enolacetone intermediate from free protons (Figure 4). In this way, 2-KPCC, while a relatively slow enzyme, is able to repurpose the disulfide-cleaving platform of typical DSORs like GR for carboxylase chemistry. This model is fully consistent with all of our data, enzymatic precedent, and chemical logic.

EXPERIMENTAL PROCEDURES
Expression and purification of WT and mutant 2-KPCC. E. coli BL21(DE3)pLysS cells were transformed with the pBAD plasmid harboring the wt or corresponding mutant 2-KPCC gene from Xanthobacter autotrophicus Py2, plated on LB agar + kanamycin (25 μg/mL) and grown overnight. A single colony from the plate was used to grow a 5-mL overnight culture in LB. The 5 mL of overnight culture was used as the inoculum for a 500 mL baffled flask containing 500 mL of ZYP-Rich media +kanamycin (25 μg/mL). Cells were grown at 37°C with agitation at 225 rpm until the OD$_{600}$ reached 0.6 – 1.0. The temperature was reduced to 25°C, arabinose was added to 0.02%, and the cells
were grown for an additional 16-18 hours. Cells were pelleted by centrifugation, frozen, and stored at -80°C.

The cell pellet was resuspended in 4 volumes of Lysis Buffer (20mM Tris pH 8.0, 300 mM NaCl, 5 mM imidazole, 1 mM PMSF) and thawed at 30 °C. All subsequent treatments were performed on ice or at 4 °C. Resuspended cells were lysed via multiple rounds of sonication (Branson Ultrasonifier). Cell lysates were clarified via centrifugation at 95,000 x g for 45 min. Clarified lysates were loaded onto a Ni-NTA resin column via gravity, washed with Lysis Buffer, and eluted using a 0-0.4 M imidazole gradient in Lysis Buffer at 2 mL/min. The eluted 2-KPCC was diluted 5-fold into Buffer A (20 mM Tris-Cl pH 6.5, 10% w/v glycerol), applied to a DEAE-sepharose ion-exchange column (GE biosciences), and eluted using a 0-1M NaCl gradient in Buffer A. Fractions were screened using SDS-PAGE. Protein was pooled and dialyzed in 20 mM Tris-Cl pH 7.4, 10% glycerol and 200 mM NaCl. Protein was pressure concentrated using 10 kDa MWCO filters (Millipore).

Total protein concentration was determined using a BCA protein assay (Thermo Scientific) while flavin concentration was determined from its UV-vis absorbance at 450 nm using an $\varepsilon_{450} = 11,828$ M$^{-1}$ cm$^{-1}$. All concentrations of protein cited in the text refer to flavin-containing protein.

**Measurement of $K_d$ for NADP$^+$ by fluorescence titration.** Fluorescence excitation at 460 nm of 2-KPCC gives rise to large fluorescence band between 480-600 nm, with a maximal signal at 525 nm. Titration of 5 μM wt, CysINTAla, or CysINTAla/F501H (100 mM Phosphate buffer pH 6.5 or 100 mM CAPS buffer pH 9.5) with NADP$^+$ resulted in loss of the fluorescence signal at 525 nm. Plots of the change in fluorescence intensity ($\Delta$fluor) versus NADP$^+$ concentration were indicative of multi-site binding and were fit with a sum of two Langmuir isotherms to determine $K_d$ values:

$$\Delta\text{fluor} = \frac{[L]}{K_{D1}+[L]} + \frac{[L]}{K_{D2}+[L]}$$

(1)

Where [L] is the free (unbound) ligand concentration.

**Monitoring UV/vis changes in the flavin species in WT and mutant 2-KPCC as a function of pH.** All pH titrations were carried out anaerobically (Coy anaerobic chamber) using an Agilent 8453 spectrometer with diode array detection. Titration measurements for the NADPH reduced wt, BES treated, and CysINTAla variant were conducted using a procedure similar to that described in Kofoed et al. (17). For the reduction of wt 2-KPCC, 1 eq. of deoxygenated NADPH was added to the stock solution of protein prior to its introduction into deoxygenated GTP buffer at the appropriate pH.

**Steady state kinetic measurements.** 2-KPCC reactions were carried out anaerobically in a Coy chamber by monitoring NADPH consumption at 340 nm on an Agilent 8453 spectrometer with diode array detection. Protein and buffer solutions were made anaerobic using a double-manifold Schlenk line with alternating cycles of argon gas purging and evacuation. Stock solutions of NADPH and 2-KPC were made from solid powders in N$_2$-purged buffer; in the absence of CO$_2$, the product of the reaction is acetone. All reactions were initiated by addition of enzyme. Reactions were monitored at 340 nm for 150 s at 5 s intervals. The initial linear portion of the change in absorbance traces was fit via linear regression analysis (NADPH $\varepsilon_{340} = 6225$ μM$^{-1}$ cm$^{-1}$) to
determine the initial rate of reaction \( (v_i) \). For each 2-KPC concentration, reactions were carried out in at least triplicate and averaged. The average rate was plotted as a function of [2-KPC]. Data were fit to the Michaelis Menton model:

\[
\frac{v_i}{[E]} = \frac{k_{\text{cat}}[S]}{K_m + [S]}
\]

(2)

where \([E]\) is the concentration of 2-KPCC, \([S]\) is the [2-KPC], \(k_{\text{cat}}\) is theoretical maximal turnover rate at saturating substrate, and \(K_m\) is the substrate concentration at half the value of \(k_{\text{cat}}\). For data that exhibited substrate inhibition, the following model was used:

\[
\frac{v_i}{[E]} = \frac{k_{\text{cat}}[S]}{K_m + [S] + [S]^2/K_I}
\]

(3)

\(K_I\) describes the concentration of substrate the causes inhibition of rate to half the theoretical maximal rate \((k_{\text{cat}})\) in the absence of any inhibition.

Oxidative half reactions monitored as a function of [2-KPC] and pH in the absence or presence of CO\(_2\). The reactions were conducted as described above with the pH varied from 6.5 – 9.5 (200 mM Glycine-Tris-Phosphate buffer with 200 mM NaCl) in 0.5 pH unit increments. All reactions were carried out on a 1 ml scale with addition of NADPH from a stock of 10 mM to achieve a final concentration of 100 \(\mu\)M. Stock solutions of 2-KPC (10 mM and 100 mM) were made by dissolving solid powder in the working buffer pH and were then added to the reaction cuvette prior to enzyme addition to achieve final concentrations ranging from 100 – 4000 \(\mu\)M. For reactions carried out in the presence of CO\(_2\), 60 mM KHCO\(_3\) was added to each buffer immediately prior to use, consistent with prior work (8). As a control, a plot of specific activity as a function of pH was measured at 10, 20, and 50 mM KHCO\(_3\), confirming that 60 mM KHCO\(_3\) indeed supplies a saturating concentration of CO\(_2\) across the pH range used (pH 6.5-9.5).

Observing the single-turnover reductive and oxidative half reactions by stopped flow. Data were measured using a KinetAssyst stopped flow spectrometer (Hi-Tech Scientific) in single-mixing mode with either diode array or single wavelength photomultiplier detection at 460 or 600 nm. The spectrometer was made anaerobic by overnight incubation with protocatechuate dioxygenase (PCD) and its substrate, protocatechuic acid (PCA), or via incubation of the stopped flow sample handling unit with a 2 mM dithionite solution which was subsequently removed from the lines with large amounts deoxygenated buffer. 2-KPCC stock solutions were made anaerobic using a double-manifold Schlenk line with alternating cycles of argon gas purging and evacuation. For monitoring the reductive half reaction with NADPH, solutions of 2-KPCC were diluted to working concentrations in a 200 mM Glycine-Tris-Phosphate buffer with 200 mM at pH 6.5 – 9 and sealed in an airtight tonometer that interfaced with the stopped flow sample handling unit. Deoxygenated buffer or NADPH solutions were prepared in the anaerobic chamber, sealed in gastight syringes, and then introduced into the sample handling unit for reaction. For reactions monitoring the oxidative half reaction, the protein was titrimetrically reduced with one equivalent of NADPH prior to being sealed in the airtight tonometer. 2-KPCC solutions were prepared in the anaerobic chamber using anaerobic buffer, sealed in gastight syringes and introduced to the sample handling unit for reaction. Data were measured at varying
time points and fit using the Kinetic Studio (Hi-Tech Scientific) software to exponential decay functions to determine rate constants ($k_{obs}$). For each experimental condition, all data were measured in at least triplicate and averaged.

**Proton inventory through solvent isotope effects.** All reactions were conducted under N$_2$ in an anaerobic chamber (COY). Solutions of 100 mM GTP buffer + 100 mM NaCl at pH/D of 7 were made made in either $^1$H$_2$O or $^2$H$_2$O (Acros Organics, 99.8 % $^2$H) from Tris-base, K$_3$PO$_4$, glycine, and NaCl. The resulting solutions were brought to a p$^1$H/2$^2$H of 7.0 (p$^2$H = pH* + 0.41, where pH* is the apparent pH measured using a standard glass electrode) via addition of either $^1$HCl or $^2$HCl (Acros Organics, 99% $^2$H). Stock solutions of 500 mM DTT and 500 mM BES were prepared in a 50:50 mix of $^1$H$_2$O/$^2$H$_2$O. Variable isotopic buffer solutions were made from a mixture of $^1$H$_2$O and $^2$H$_2$O buffer, with the percentage of $^2$H$_2$O varied at either: 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, or 100 %. DTT and BES from the concentrated stocks were added to each solution to give final concentrations of 5 mM BES and 10 mM DTT. Enzyme from a concentrated stock was added to the varied percentage $^1$H$_2$O/$^2$H$_2$O solutions. A control sample made in 100 % $^1$H$_2$O that lacked BES was run in parallel. In all cases the total volume of enzyme, DTT, and BES added was less than 4 % the total reaction volume and hence did not heavily influence the solution $^1$H/$^2$H composition. The samples were allowed to incubate at 20 °C for 4 hours. To remove the BES, samples were treated with DOWEX resin as previously (17). Each sample was assayed for activity via addition of the BES treated enzyme to a 5 mM 2-KPC and 0.1 mM NADPH solution made in 100 mM GTP buffer at pH 7. The initial rates of NADPH consumption were monitored at 340 nm and compared with the sample control. Triplicate technical replicates were carried out for each ratio $^1$H/$^2$H sample for two experiment replicates.

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**REFERENCES**


**FOOTNOTES**

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2. The abbreviations used are: DSOR, disulfide oxidoreductases; 2-KPCC, NADPH-2-ketopropyl-coenzyme M oxidoreductase/ carboxylase; RuBisCO, ribulose-1,5-bisphosphate carboxylase/oxygenase; Cys<sub>CT</sub>, charge tra H<sub>2</sub>O er thiol; Cys<sub>INT</sub>, interchange thiol; GR, glutathione reductase; GSSG, glutathione disulfide; GSH, glutathione; Cys<sub>INT</sub>-S-SG, mixed disulfide intermediate; Cys<sub>CT</sub>-S<sub>INT</sub>Cys, oxidized cysteine disulfide; 2-KPC, 2-ketopropyl-coenzyme M or [2-(2-ketopropylthio)ethanesulfonate]; BES, bromoethanesulfonate; PCD, protocatechuate dioxygenase; PCA, protocatechuc acid.
Figure 1. Canonical steps by which the active site of DSORs are reduced, yielding the reactive form of the active site. The oxidized form of the enzyme (1) rapidly binds NADPH (2). A hydride is transferred from NADPH to the flavin (3), rendering its characteristic yellow chromophore colorless. The flavin subsequently transfers two electrons to the nearby disulfide in a process catalyzed by the active site histidine, yielding a C4a-flavin-CysCT covalent intermediate (4). The covalent species rapidly tautomerizes to yield the catalytically active CT complex (6), which is stabilized by the active site HisH+. Stopped flow kinetics studies of the reductive half reaction of glutathione reductase identified two phases, diagrammed by the arrows. In the first phase, the oxidized flavin is converted to the reduced form (3). In the second, slightly slower phase, the flavin reoxidizes to yield the reactive CT species (5). Second order rate constants for each phase are given for the WT GR. Values for the active site histidine mutant (H439A) are given in parentheses (9). Notably, formation of the reduced flavin is only clearly observed for the H439A mutant, due to >10-fold slowing of the reoxidation of the flavin. Rate constants measured for the analogous two phases in 2-KPCC were orders of magnitude smaller: $k = 27 \pm 3 \text{ s}^{-1}$ and $10 \pm 2 \text{ s}^{-1}$ (pH 6.5, 25 °C) respectively (see text).
Figure 2. Possible reactive protonation states for the 2-KPCC active site. The reduced, reactive form of 2-KPCC at neutral pH could be either an EH$_2$ or EH- form. The E$^{2-}$ form (D) is considered unlikely to be biologically relevant because pKa2 is potentially too high for a substantial amount of this species to accrue. Moreover, because the spectrum for reduced 2-KPCC exhibits no CT band, only the non-CT species are possible candidates, and we therefore rule out both species (D) and (B), leaving (A) and (C) as possible models for the active site. The EH- form in (C) stabilizes a nucleophilic thiolate, similar to the proposed reactive form for thioredoxin active sites (32). Note: the canonical reactive form for DSORs resembles form (B), but with HisH$^+$ available to stabilize the flavin-CysCT charge transfer interaction. Since both the histidine and Cys$_{INT}$ bear protons, it is an EH$_2$ state.
Figure 3. Structures of 2-KPCC and a representative DSOR (glutathione reductase, PDB ID: 1GRE). (A) Structure of the CO$_2$-bound 2-KPCC homodimer (PDB ID: 3Q6J) with subunits in green and cyan cartoon. The FAD (red), NADP$^+$ (yellow), cysteine dithiol (orange) are shown as sticks. The Phe501 side chain is shown as sticks in a dark blue. 2-KPCC-bound CO$_2$ is shown in magenta. (B) The cofactors and catalytically important side chains from one of the active sites in (A) are highlighted alone and on a magnified scale. The active sites of (C) glutathione reductase (GR) (PDB ID:1GRE) and (D) 2-KPCC in their oxidized, disulfide forms are compared (carbon grey; nitrogen blue; sulfur orange; oxygen red). The conserved His-Glu in GR are replaced by Phe and His, respectively, in 2-KPCC.
Figure 4. Proposed oxidative half reaction for 2-KPCC under carboxylating conditions. Note that the enolacetone intermediate created in the first step of the 2-KPCC reaction can react with any available protons to form the off-pathway product acetone. Restricting water or other sources of protons from the active site is therefore critical for reaction fidelity. At the same time, the carboxylation reaction as proposed does not require the entry or loss of protons from the active site, and instead uses Cys<sub>CT</sub> as an internal acid-base.
Figure 5. The slow reductive half reaction of 2-KPCC has flavin reduction and reoxidation steps followed by photoreduction. The reaction between anaerobic 15 μM 2KPCC (red trace) and 10 eq of NADPH at pH 6 (100 mM Tris-Cl) results in large changes to the flavin spectrum over time. After 100 ms (blue) and by 1s (orange trace) the intensity of the flavin absorbance decreases by ~2-fold. This is followed by an increase and slight blue shift in the flavin absorbance, maximizing at 70 s (green). Under the bright light used for diode array detection, photoreduction of the oxidized flavin, accompanied by increases in absorbance at 550-700 nm, occurs subsequently over longer time scales, indicative of formation of a flavin semiquinone (200 and 700 s; purple and black traces).
Figure 6. The reductive half reaction in 2-KPCC is slow relative to typical DSORs and occurs in multiple observable phases. (A) The reaction of 15 μM 2-KPCC at pH 6.5 (100 mM phosphate buffer) with 10 eq of NADPH monitored at 460 nm by stopped flow exhibited three kinetic phases. The first two phases correspond to loss and gain of absorptivity by the flavin chromophore (flavin reduction and reoxidation) and were fit to single exponential curves to yield $k = 27 \pm 3 \text{ s}^{-1}$ and $k = 10 \pm 2 \text{ s}^{-1}$ (errors are ± 1 standard deviation from 3 measurements). The third phase corresponds to subsequent, still undefined changes in the chromophore as it reaches its final form. Note that the use of monochromatic light avoids the photoreduction phase, which obscures the final phase when white light excitation is used (Figure 5). (B) Reaction of 15 μM 2-KPCC at varying pH (6.5-9.5) with 10 eq of NADPH was monitored at 460 nm by stopped flow. At pH 6.5 (red), 7 (orange), and 7.5 (gold) three kinetic phases are observed. At pH 8 (green), 8.5 (light blue), 9 (dark blue), and 9.5 (purple) the two phases have converged.
Figure 7. **2-KPCC does not form a C4a-adduct at low pH, and converts to a CT species at very high pH.** Changes in the UV/visible spectrum for CYS\textsubscript{INT}Ala 2-KPCC in the presence of bound NADP\(^+\) were monitored as a function of pH over pH 6.5-11 (20 μM 2-KPCC, 100 μM NADP\(^+\), 100 mM glycine-Tris-phosphate buffers). The pH 6.5 spectrum is shown in gold, and the pH 11 spectrum in orange. Intermediate spectra are in grey. In typical DSORs, the acidic form of the active site in the presence of NADP\(^+\) is the C4a-covalent adduct, characterized by a single intense band centered at 380 nm. This converts to the CT species (apparent pK\(_a\) = 6.8), which is stabilized by the active site HisH\(^+\). The low/neutral pH species for 2-KPCC clearly does not share the characteristic UV/vis spectrum of a C4a-adduct, making its electronic and protonation state unclear. Inset: An apparent pK\(_a\) of 9.4 ± 0.1 corresponding to formation of the CT species was determined by plotting absorbance at 570 nm vs pH and fitting to a sigmoidal curve.
Figure 8. The inactivation of 2-KPCC by BES is pH dependent and reflects changes to the protonation state of Cys\textsubscript{INT}. 2-KPCC in the presence of 10 mM DTT was incubated with BES for 80 min at pH values ranging from 5.6 – 9.5. The BES was removed and the protein was assayed for 2-KPC carboxylation activity (pH 7.5, 100 μM NADPH, 60 mM bicarbonate, and 1 mM 2-KPC). The specific activity of the carboxylation reaction relative to a control sample showed increasing BES inactivation as the pH is raised. The data were fit with a sigmoidal curve yielding a pK\textsubscript{a} of 8.1 ± 0.1, ascribed to Cys\textsubscript{INT}. 
Figure 9. Plotting the effect of pH on $k_{\text{cat}}/K_M[2\text{-KPC}]$ indicates a pKa of 7.5 that is attributed to Cys$_{\text{INT}}$. pH-rate profiles were measured with 2-KPCC and variable [2-KPC] for the reaction with $H^+$ to produce acetone (red circles) or $CO_2$ to make acetoacetate (blue circles). Data were fit to a two-pKa model. The lower pKa in each case is 7.5 (acetone product) and 7.7 (acetoacetate product).
Figure 10. A proton inventory experiment indicates that no protons enter or exit the 2-KPCC active site as the reduced form cross-links with a substrate analog. The rate of the irreversible crosslinking reaction between BES and reduced 2-KPCC was monitored as a function of the fraction of D$_2$O in the buffer. The complete independence of the crosslinking rate indicates that protons are not gained or lost during the crosslinking step.
Figure 11. The rate of the oxidative half reaction between reduced 2-KPCC and 2-KPC, with and without excess CO₂, was monitored as a function of [2-KPC]. 2-KPCC (20 μM, 50 mM Tris, pH 7.4) was reduced with a slight molar excess of NADPH (24 μM) and then rapidly mixed with 50, 100, or 200 μM 2-KPC in the presence (red circles) or absence (blue circles) of excess CO₂ (supplied as 60 mM KHCO₃). The subsequent reactions were monitored by stopped flow UV/Vis spectroscopy. The data were fit to double exponential functions corresponding to the entire oxidative half reaction followed by photoreduction. The rate constant for the initial reaction phase varied linearly with [2-KPC] in each case, yielding rate constants $k = 6.7 \times 10^{-4}$ μM s⁻¹ (CO₂ present) and $k = 1.1 \times 10^{-4}$ μM s⁻¹ (CO₂ absent).