The reactive form of a C–S bond–cleaving, CO₂-fixing flavoenzyme

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NADPH:2-ketopropyl–coenzyme M oxidoreductase/carboxylase (2-KPCC) is a bacterial disulfide oxidoreductase (DSOR) that, uniquely in this family, catalyzes CO₂ 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 CO₂. The reductive half-reaction of 2-KPCC was kinetically and spectroscopically similar to that of a typical DSOR, GSH 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 pKₐ 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 chemosynthetic microbes. In phototrophs, the first step of CO₂ fixation is catalyzed by ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO),2 the most abundant enzyme on earth (1). A variety of processes that fix CO₂ or its hydrated form (bicarbonate, HCO₃⁻) in nonphotosynthetic 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 FAD and cysteine-disulfide–containing oxidoreductases (DSORs) that are best known for reducing disulfide (GSH 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 (Fig. 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) in which the cysteine proximal to the FAD (known as the charge-transfer thiol, CysS₄SH) participates in a charge-transfer interaction with the oxidized flavin (CysS₄C–SH ... FAD). This CT species is stabilized by the charge on the active site acid–base histidine (Figs. 2 and 3). The cysteine distal to the FAD, known as the interchange thiol (CysS₄S–SH), 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 GSH reductase (GR), a well-characterized model DSOR, GSSG is reductively cleaved by CysS₄NT and protonated by HisH⁺ to form one molecule of GSH and a covalent, mixed disulfide intermediate (CysS₄NTS–SG). Protonation of the covalent intermediate then yields the second equivalent of GSH and allows the oxidized cysteine disulfide (CysS₄NTS–Cys) 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 enolacetoanone anion that nucleophilically attacks enzyme-bound CO₂ to form the new carbon–carbon bond of acetoacetate (Fig. 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 a loss of nearly all catalytic function in GR (4, 7); however, the native residue at this position in 2-KPCC is a phenylalanine (Phe501) (Fig. 3D).

We previously showed that substitution of a histidine at this position (F501H) 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

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**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 HisH1101. Stopped-flow kinetics studies of the reductive half-reaction of GSH 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, because of 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: \( k = 27 \pm 3 \text{ and } 10 \pm 2 \text{ s}^{-1} \) (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 EH2 or EH form. The EH2 form (D) 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 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 that the canonical reactive form for DSORs resembles form B, but with HisH1101 available to stabilize the flavin–CysCT charge transfer interaction. Because both the histidine and CysINT bear protons, it is an EH2 state.
then slowly grew back in intensity, corresponding to transient flavin reduction (Fig. 5). The peak at 460 nm, where NADPH was saturating (15 μM), was 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 (Fig. S2).

The transient reduction and reoxidation of the flavin were most distinct at low pH. To visualize each of these events clearly and without interference from the final photoreductive phase, the reductive half-reaction was monitored again with UV-visible absorption at a single wavelength (460 nm) at pH 6.5 (Fig. 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 $k = 13 \pm 2$ 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 H439A mutant of GR (Fig. 1). We conclude that the presence of a hydrophilic residue at this position (GR H439A or (2-KPCC Phe$^{501}$)) at this position has a broadly similar effect (Fig. 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 (Fig. 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 GSH in its two-electron-reduced state with NADP+ bound. This form (designated as EH2) 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 pK$a$ on Cys$^{\text{INT}}$ to near 7.5 (10). This primes Cys$^{\text{INT}}$ to lose a proton prior to nucleophilic attack on GSH (Fig. 2). Below pH 7.5, the EH3+ 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 EH3+ 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 pK$a$ of Cys$^{\text{CT}}$ was previously measured in the absence of NADP+ using the 2-KPCC Cys$^{\text{INT}}$ variant (C82A or Cys$^{\text{INT}}$ Ala), which has just one titratable active-site cysteine. A pK$a > 9.0$ was determined, reflecting the hydrophobic environment of this active site (Fig. 3) (17). NADP+ is critical both for stabilizing the C4a adduct and for catalysis (16). We therefore monitored changes in the UV-visible spectrum of the Cys$^{\text{INT}}$ Ala–NADP+ complex of 2-KPCC as a function of pH (see Fig. 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

**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 (Fig. 1) (4, 10). This is the catalytically competent form of the enzyme that binds to and reduces substrate. The reductive half is rate-limiting in DSOrs and may be orders of magnitude slower than the analogous reductive half-reaction of GR (4) and are much closer to the rate constants measured for the H439A mutant of GR (Fig. 1). We conclude that the presence of a hydrophilic residue at this position (GR H439A or (2-KPCC Phe$^{501}$)) at this position has a broadly similar effect (Fig. 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 (Fig. 6B) (4). The source of the pH effect is not clear.
transfer species only formed at high pH ($pK_a = 9.4 \pm 0.1$), despite the presence of the nearby positively charged nicotineamide ring (Fig. 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 (Fig. 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 (Fig. 3) and the available data suggest two possible descriptions for the reactive form (4) (Fig. 2). The first is an EH$_2$ species in which both CysINT and CysCT are protonated. However, the immediate environment around CysINT, dominated by Phe$^{501}$, is highly hydrophobic and devoid of basic residues. Such an environment is not expected to support facile proton loss by CysINT. Prior work with DSORs suggests CysINT must be deprotonated to be an effective nucleophile for breaking S–S bonds (9). Hence, we expect CysINT 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 CysINT and shared in a close hydrogen-bonding interaction with CysCT (9). 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 RNase HI (22). Sharing the proton between the two sulfur atoms in thioredoxins (enzymes that 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 μM) 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 CysINT in 2-KPCC as well as in reduced enzyme. The C–Br bond in BES is a surrogate for the C–S bond in 2-KPC, and Br⁻ is the leaving group (23). Only deprotonated CysINT is expected to cross-link with BES (23); hence, the pH dependence of 2-KPCC inactivation serves as a functional measure of the CysINT–SH pKᵰ. The data in Fig. 8 showed that formation of an irreversible cross-link increases at elevated pH with a pKᵰ = 8.1 ± 0.1, which we attribute to the CysINT–SH.

Second, the steady-state kinetics of the 2-KPCC–catalyzed reaction between NADPH and 2-KPC in the presence and absence of CO₂ were monitored as a function of pH values ranging from 5.6 to 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 pKᵰ of 8.1 ± 0.1, ascribed to CysINT.

The two pH-rate profiles in Fig. 9 share the pKᵰ of 7.5 (acetone product) and 7.7 (acetocetoate product). We interpret the increase in reactivity to the pKᵰ of CysINT (Fig. 9). The decrease in kcat/Km[2-KPC] above this pH is most likely due to enzyme inactivation.

The enolacetone preferentially reacts with CO₂ when it is saturating to form acetocetoate (8), as shown in prior work. The parameter kcat/Km[2-KPC] under CO₂-saturated conditions has the same bell shape for its pH-rate profile and the same pKᵰ values as in the absence of CO₂. 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 CO₂.

The two pKᵰ values determined for CysINT in the BES-inactivation experiments (Fig. 8), which we again tentatively ascribe to the CysINT proton. A hallmark of systems with side-chain–shared protons is the observation of pKᵰ values shifted downwards from expected values (9). For a cysteine buried in a hydrophobic environment (25), we expect the pKᵰ to be above the free-cysteine value of 8.5. Indeed, the pKᵰ for CysCT in the CysINT-Ala mutant is elevated to above 9.4. The pKᵰ of 7.5 for CysINT, despite the absence of any general base or negative charge-stabilizing group, suggests the involvement of CysCT in a shared hydrogen-bonding interaction.

![Figure 7](image7.png) 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 CysINTAla 2-KPCC in the presence of bound NADP⁺ were monitored as a function of pH over 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 is in orange. Intermediate spectra are in gray. 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 pKᵰ = 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-visible spectrum of a C4a adduct, making its electronic and protonation state unclear.

![Figure 8](image8.png) The inactivation of 2-KPCC by BES is pH-dependent and reflects changes to the protonation state of CysINT. 2-KPCC in the presence of 10 mM DTT was incubated with BES for 80 min at pH values ranging from 5.6 to 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 pKᵰ of 8.1 ± 0.1, ascribed to CysINT.

![Figure 9](image9.png) Plotting the effect of pH on kcat/Km[2-KPC] indicates a pKᵰ of 7.5 that is attributed to CysINT. pH-rate profiles were measured with 2-KPCC and variable [2-KPC] for the reaction with H⁺ to produce acetone (red circles) or CO₂ to make acetoacetate (blue circles). The data were fit to a two-pKᵰ model. The lower pKᵰ in each case is 7.5 (acetone product) and 7.7 (acetocetoate 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 cross-linking reaction between BES and reduced 2-KPCC was monitored as a function of the fraction of D2O in the buffer. The complete independence of the cross-linking rate indicates that protons are not gained or lost during the cross-linking step.

Notably, the values of $k_{\text{cat}}/K_m[2\text{-KPC}]$ are 1–2 orders of magnitude larger for the carboxylation of enolacetone than for its protonation, across the pH range. The reasons for this steady-state turnover with CO2 are not clear. However, we note that the concentration of CO2 under our experimental conditions, where CO2 is introduced by adding a large excess of KHCO3 (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 mM as the pH varies from 6.5 to 9.5). We postulate that the faster observed turnover with CO2 is due to the relatively higher concentration of CO2 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 concentrations of 2-KPC, with and without excess CO2, was monitored as a function of [2-KPC]. 2-KPC (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-visible 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 $k = 6.7 \times 10^{-4} \mu M s^{-1}$ (CO2 present) and $k = 1.1 \times 10^{-4} \mu M s^{-1}$ (CO2 absent).

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 (Fig. 11), yielding rate constants $k = 6.7 \times 10^{-4} \mu M s^{-1}$ (CO2 present) and $k = 1.1 \times 10^{-4} \mu M s^{-1}$ (CO2 absent). As with $k_{\text{cat}}/K_m[2\text{-KPC}]$, the presence of CO2 appeared to increase the rate of the oxidative half-reaction, although again, the concentration of CO2 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 $k = 0.14 s^{-1}$ (CO2 present) and $k = 0.022 s^{-1}$ (CO2 absent). By comparison, the analogous rate constant for the GR oxidative half-reaction was many orders of magnitude faster: 3900 s$^{-1}$ in saturating GSH (25 °C) (4). The reductive half-reaction of 2-KPC was also relatively faster (rate constants $k = 27 \pm 3$ 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-KPCC.

**Discussion**

2-KPCC is a member of a large class of oxidoreductases that contain FAD and a cysteine-disulfide. Unique among this family, 2-KPCC 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 CO2 into biomass (Fig. 4F). At the same time, 2-KPCC effectively prevents H+ from reaching the enolacetone intermediate and forming unwanted acetone. We hypothesized that the hydrophobic active site of 2-KPCC, which lacks a conserved histidine (Fig. 3), may be important for both C–S bond cleavage and carboxylation fidelity.

Our results here suggest that the hydrophobic active site of 2-KPCC disfavors forming the flavin–Cys$_{\text{CT}}$ charge transfer

![Figure 10](image1.png)

![Figure 11](image2.png)
intermediate, although this is the reactive form of the active site in all other well-studied DSORs (4, 27–30). In these enzymes, the active site CysINT 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 (Fig. 4), however, appears to favor formation of the protonated rather than the carboxylated product, according to prior work with the F501H 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 CysINT? 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 CysINT in reduced 2-KPCC, we can now propose a mechanism that also stabilizes bound CO₂. This model is reminiscent of the shared-proton model proposed for thio-redoxins (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 CysCT and CysINT in reduced 2-KPCC, we can now propose a mechanism for the oxidative half-reaction in which the acid–base functions of CysINT in a hydrophobic environment that also stabilizes bound CO₂. This model is reminiscent of the shared-proton model proposed for thio-redoxins (9), another family of enzymes that react via a cysteine disulfide.

In this way, 2-KPCC, although 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 medium + kanamycin (25 μg/ml). Cells were grown at 37 °C with agitation at 225 rpm until the A₆₀₀ 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 h. The cells were pelleted by centrifugation, frozen, and stored at −80 °C.

The cell pellet was resuspended in 4 volumes of lysis buffer (20 mM Tris, pH 8.0, 300 mM NaCl, 5 mM imidazole, 1 mM phenylmethylsulfonyl fluoride) and thawed at 30 °C. All subsequent treatments were performed on ice or at 4 °C. The resuspended cells were lysed via multiple rounds of sonication (Branson Ultrasonifier). Cell lysates were clarified via centrifugation at 95,000 g for 45 min. Clarified lysates were loaded onto an nitrilotriacetic–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 0–1 M 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 molecular mass cutoff filters (Millipore). Total protein concentration was determined using a BCA protein assay (Thermo Scientific), whereas flavin concentration was determined from its UV-visible absorbance at 450 nm using an ε₄₅₀ of 11,828 M⁻¹ cm⁻¹. All concentrations of protein cited in the text refer to flavin-containing protein.

**Measurement of Kₐ for NADP⁺ by fluorescence titration**

Fluorescence excitation at 460 nm of 2-KPCC gives rise to large fluorescence band between 480 and 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 (Δfluor) versus NADP⁺ concentration were indicative of multisite binding and were fit with a sum of two Langmuir isotherms to determine Kₐ values.

\[
\Delta \text{fluor} = \frac{[L]}{K_{d1} + [L]} + \frac{[L]}{K_{d2} + [L]} \quad \text{(Eq. 1)}
\]

where [L] is the free (unbound) ligand concentration.
**CO$_2$-fixing flavoenzyme**

**Monitoring UV-visible 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 by 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. The 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 $e_{340} = 6225 \, \mu M^{-1} \, cm^{-1}$) 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]. The data were fit to the Michaelis–Menten model,

$$v_i = \frac{k_{cat}[S]}{K_m + [S]}$$  \hspace{1cm} (Eq. 2)

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

$$v_i = \frac{k_{cat}[S]}{K_m + [S] + \frac{[S]^2}{K_i}}$$  \hspace{1cm} (Eq. 3)

$K_i$ describes the concentration of substrate the causes inhibition of rate to half the theoretical maximal rate ($k_{cat}$) 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 carried as described above with the pH varied from 6.5 to 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 mixture of 1H$_2$O and 2H$_2$O (Acros Organics, 99.8% 2H) 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 1HCl or 2HCl (Acros Organics, 99% 2H). Stock solutions of 500 mM DTT and 500 mM BES were prepared in a 50:50 mix of 1H$_2$O/2H$_2$O. Variable isotopic buffer solutions were made from a mixture of 1H$_2$O and 2H$_2$O buffer, with the percentage of 2H$_2$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 1H$_2$O/2H$_2$O solutions. A control sample made in 100% 1H$_2$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

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**Observing the single-turnover reductive and oxidative half-reactions by stopped flow**

The 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 and its substrate, protocatechuate acid, 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 gas-tight syringes, and 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-KPC solutions were prepared in the anaerobic chamber using anaerobic buffer, sealed in gas-tight syringes, and introduced to the sample-handling unit for reaction. The data were measured at varying time points and fit using the Kinet 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 Lab Products). Solutions of 100 mM GTP buffer + 100 mM NaCl at pH/D of 7 were made in either 1H$_2$O or 2H$_2$O (Acros Organics, 99.8% 2H) 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 1HCl or 2HCl (Acros Organics, 99% 2H). Stock solutions of 500 mM DTT and 500 mM BES were prepared in a 50:50 mix of 1H$_2$O/2H$_2$O. Variable isotopic buffer solutions were made from a mixture of 1H$_2$O and 2H$_2$O buffer, with the percentage of 2H$_2$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 1H$_2$O/2H$_2$O solutions. A control sample made in 100% 1H$_2$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 h. 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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