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

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Bennett R. Streit, Jenna R. Mattice, Gregory A. Prussia, John W. Peters, and Jennifer L. DuBois

From the Department of Chemistry and Biochemistry, Montana State University, Bozeman, Montana 59715-3400 and the Institute of Biological Chemistry, Washington State University, Pullman, Washington 99163

Edited by Ruma Banerjee

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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 pHₐₜ 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 photosynthots, the first step of CO₂ fixation is catalyzed by ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBiCO),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, Cys₅C₇SH) participates in a charge-transfer interaction with the oxidized flavin (Cys₇C₅– →... → FAD). This CT species is stabilized by the charge on the active site acid–base histidine (Figs. 2 and 3C). (4) The cysteine distal to the FAD, known as the interchange thiol (Cys₉ᵢNTSH), has its pHₐₜ 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 CysᵢNT and protonated by HisH⁺ to form one molecule of GSH and a covalent, mixed disulfide intermediate (CysᵢNTS–SᵢNTCys). Protonation of the covalent intermediate then yields the second equivalent of GSH and allows the oxidized cysteine disulfide (CysᵢNTS–SᵢNTCys) 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 that nucleophilically attacks enzyme-bound CO₂ to form the new carbon–carbon bond of acetoacetate (Fig. 4). In the absence of CO₂ and disulfide; 2-KPC, 2-ketopropyl–coenzyme M or 2-(2-ketopropylthio)ethanesulfonate; BES, bromoethanesulfonate; PDB, Protein Data Bank.

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This article contains Figs. S1–S4.

1 To whom correspondence should be addressed. Tel.: 406-994-2844; E-mail: jennifer.dubois1@montana.edu.

2 The abbreviations used are: RuBiCO, ribulose-1,5-bisphosphate carboxylase/oxygenase; DSOR, disulfide oxidoreductases; 2-KPCC, NADPH:2-ketopropyl–coenzyme M oxidoreductase/carboxylase; Cys₅C₇, charge transfer thiol; CysᵢNT, interchange thiol; GR, glutathione reductase; CysᵢNTS–SᵢNT, mixed disulfide intermediate; Cys₅C₇–SᵢNTCys, oxidized cysteine.
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.

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 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 ± 3 and 10 ± 2 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 EH⁻ form (D) is considered unlikely to be biologically relevant because pK₂ 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 HisH⁺ 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 where NADPH was saturating (15 μM NADPH, pH 6, 25°C) (Fig. S1). A clear loss in absorptivity was observed in the characteristic flavin peak at 460 nm, corresponding to transient flavin reduction (Fig. 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 (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 photoelectrodative 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 $k = 13 \pm 2 \text{s}^{-1}$, 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 H439A mutant of GR (Fig. 1). We conclude that the presence of a hydrophobic residue at this position (GR H439A or (2-KPCC Phe501)) 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 E_H2) has two protons in the active site. One proton resides on the conserved histidine (His_H⁺) and the other on CysINT, (4). The charge on His_H⁺ stabilizes a charge transfer interaction between the flavin and CysCT and lowers the pKₐ on CysINT near 7.5 (10). This primes CysINT to lose a proton prior to nucleophilic attack on GSH (Fig. 2). Below pH 7.5, the EH₃⁺ form predominates, in which the CysCT 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 CysINT. 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 pKₐ of CysCT was previously measured in the absence of NADP⁺ using the 2-KPCC CysINT variant (C82A or CysINT Ala), which has just one titratable active-site cysteine. A pKₐ >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 CysINTAla—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—CysCT adduct (16). Moreover, the charge 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 (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 second, oxidative half, in which the active site reduces the exogenous substrate. (11)

We monitored the reductive half-reaction of 2-KPCC (Fig. 3) by stopped flow UV-visible spectroscopy under conditions where NADPH was saturating (15 μM enzyme, 150 μM NADPH, pH 6, 25°C) (Fig. S1). A clear loss in absorptivity was initially observed in the characteristic flavin peak at 460 nm, corresponding to transient flavin reduction (Fig. 5). The peak then slowly grew back in intensity, before rapidly decaying into

**Figure 3.** Structures of 2-KPCC and a representative DSOR (GSH reductase; PDB code 1GRE). A, structure of the CO₂-bound 2-KPCC homodimer (PDB code 3Q6J) with subunits in green and cyan cartoon. The FAD (red), NADP⁺ (yellow), and cysteine dithiol (orange) are shown as sticks. The Phe501 side chain is shown as sticks in a dark blue. 2-KPCC-bound CO₂ 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 GR (C, PDB code 1GRE) and 2-KPCC (D) in their oxidized, disulfide forms are compared (carbon, gray; nitrogen, blue; sulfur, orange; and oxygen, red). The conserved His—Glu in GR are replaced by Phe and His, respectively, in 2-KPCC.
transfer species only formed at high pH (pK_a = 9.4 ± 0.1), despite the presence of the nearby positively charged nicotinamide 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 EH2 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 EH2 species in which both CysINT and CysCT are protonated. However, the immediate environment around CysINT, dominated by Phe501, 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 pKa. The data in Fig. 8 showed that formation of an irreversible cross-link increases at elevated pH with a pKa = 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 [2-KPC] and at varying pH values. When CO2 is absent, the enolacetone intermediate produced from 2-KPC cleavage reacts with H+ to form acetone. Note that varying the pH has the effect of modulating both the protonation state of CysINT and the concentration of protons available for reaction with the enolacetone from 0.32 μM (pH 6.5) to 0.32 mM (pH 9.5). Michaelis plots of initial rate versus [2-KPC] demonstrated substrate inhibition at relatively high concentrations of 2-KPC (Fig. S4) and a clear dependence on pH. The parameter kcat/Km[2-KPC] with reaction steps involving 2-KPC up to and including the one that is rate-limiting, is plotted versus pH in Fig. 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 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 acetate (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 pKa 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 pH-rate profiles in Fig. 9 share the pKa 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 values 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 CysINT is elevated to above 9.4. The pKa of 7.5 for CysINT, despite the absence of any general base or negative charge-stabilizing group, suggests the involvement of CysINT in a shared hydrogen-bonding interaction.
The oxidative half-reaction is slow relative to DSORs but faster in the presence of excess CO₂

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-KPCC (Fig. 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-KPCC was monitored over time, both in the presence and absence of saturating CO₂. 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 fitted to a 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 $k_{cat}/K_m$[2-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 (supplied as 60 mM KHCO₃). The reductive half-reaction of 2-KPCC 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–Cysᵣ charge transfer
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 CysCT in the CysINTAla variant is >9.4.

We propose here that the remarkably low pKₐ observed for CysINT, which we estimate is between 7.5 and 8.1 (Figs. 5 and 6) despite its hydrophobic environment, is due to proton sharing between CysINT and CysCT in an EH⁻ complex (Fig. 2) (9). This model is supported by proton inventory experiments, and it helps explain how CysINT 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 CysCT in 2-KPCC is instead poised to direct its electron density toward CysINT, effectively stabilizing a reactive CysINT thiolate in a hydrophobic environment that also stabilizes bound CO₂. 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 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 the active site histidine are performed instead by CysCT (Fig. 4). This likely renders the oxidative half-reaction of 2-KPCC much slower than the equivalent half-reaction in a canonical DSOR (4) (Fig. 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 CysCT and CysINT. This lowers the pKₐ of CysINT in a strongly hydrophobic environment. CysCT then acts as a built-in, internal base to retain the proton once CysINT reacts with 2-KPC, again protecting the enolacetone intermediate from free protons (Fig. 4). 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 a nickel–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 concentrated using 10-kDa molecular mass cutoff filters (Millipore). The 2-KPCC 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,

\[ \Delta \text{fluor} = \frac{[L]}{K_{d1} + [L]} + \frac{[L]}{K_{d2} + [L]} \]  
(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 Cys$_{4}$In$_{2}$Ala variant were conducted using a procedure similar to that described by Kofoid 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 $\varepsilon_{340} = 6225$ $\mu$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,

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

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

$K_i$ describes the concentration of substrate the causes inhibition of rate to half the theoretical maximal rate ($k_{\text{cat}}$) 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 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 stock of 10 mM to achieve a final concentration of 100 $\mu$M. Stock solutions of 2-KPC (10 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 to 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**

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 2 $\mu$L 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 a 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 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-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 Kinetic Studio (Hi-Tech Scientific) software to exponential decay functions to determine rate constants ($k_{\text{obs}}$). 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 1$^H$O or 2$^H$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$^1H$/p$^2H$ of 7.0 (p$^2H = p^H + 0.41$, where p$^H$ is the apparent pH measured using a standard glass electrode) via addition of either 1$^H$Cl or 2$^H$Cl (Acros Organics, 99% 2$^H$). Stock solutions of 500 mM DTT and 500 mM BES were prepared in a 50:50 mix of 1$^H$O/2$^H$O. Variable isotopic buffer solutions were made from a mixture of 1$^H$O$_2$ and 2$^H$O$_2$ buffer, with the percentage of 2$^H$O$_2$ 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 DTT and 10 mM DTT. Enzyme from a concentrated stock was added to the varied percentage 1$^H$O$_2$/2$^H$O$_2$ solutions. A control sample made in 100% 1$^H$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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References


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