Four amino acids define the CO$_2$ binding pocket of enoyl-CoA carboxylases/reductases

Gabriele M. M. Stoffel,$^{a,b}$ David Adrian Saez$^c$, Hasan DeMicri$^{d,e}$, Bastian Vögeli$^f$, Yashas Rao$^d$, Jan Zarzycki$^{a,b}$, Yasuo Yoshikuni$^i$, Soichi Wakatsuki$^{b,g,1}$, Esteban Vöhringer-Martinez$^{a,c}$, and Tobias J. Erb$^{a,b,1}$

$^a$Department of Biochemistry and Synthetic Metabolism, Max Planck Institute for Terrestrial Microbiology, D-35043 Marburg, Germany; $^b$Center for Synthetic Microbiology, D-35043 Marburg, Germany; $^c$Departamento de Fisico Quimica, Facultad de Ciencias Quimicas, Universidad de Concepcion, 4070371 Concepcion, Chile; $^d$Biosciences Division, SLAC National Accelerator Laboratory, Menlo Park, CA 94025; $^e$Stanford PULSE Institute, SLAC National Accelerator Laboratory, Menlo Park, CA 94025; $^f$Department of Energy Joint Genome Institute, Walnut Creek, CA 94598; and $^g$Structural Biology Department, Stanford University, Stanford, CA 94305

Edited by James C. Liao, Institute of Biological Chemistry, Academia Sinica, Taipei, Taiwan, and approved June 3, 2019 (received for review January 30, 2019)

Carboxylases are biocatalysts that capture and convert carbon dioxide (CO$_2$) under mild conditions and atmospheric concentrations at a scale of more than 400 Gt annually. However, how these enzymes bind and control the gaseous CO$_2$ molecule during catalysis is only poorly understood. One of the most efficient classes of carboxylating enzymes are enoyl-CoA carboxylases/reductases (Ecrs), which outcompete the plant enzyme RuBisCO in catalytic efficiency and fidelity by more than an order of magnitude. Here we investigated the interactions of CO$_2$ within the active site of Ecr from Kitasatospora setae. Combining experimental biochemistry, protein crystallography, and advanced computer simulations we show that 4 amino acids, N81, F170, E171, and H365, are required to create a highly efficient CO$_2$-fixing enzyme. Together, these 4 residues anchor and position the CO$_2$ molecule for the attack by a reactive enolate created during the catalytic cycle. Notably, a highly ordered water molecule plays an important role in an active site that is otherwise carefully shielded from water, which is detrimental to CO$_2$ fixation. Altogether, our study reveals unprecedented molecular details of selective CO$_2$ binding and C-C bond formation during the catalytic cycle of nature’s most efficient CO$_2$-fixing enzyme. This knowledge provides the basis for the future development of catalytic frameworks for the capture and conversion of CO$_2$ in biology and chemistry.

Significance

Carboxylases capture and convert CO$_2$, which makes them key enzymes in photosynthesis and the global carbon cycle. However, the question how enzymes bind atmospheric CO$_2$ is still unsolved. We studied enoyl-CoA carboxylases/reductases (Ecrs), the fastest CO$_2$-fixing enzymes in nature, using structural biology, biochemistry, and advanced computational methods. Ecrs create a highly specific CO$_2$-binding pocket with 4 amino acids at the active site. The pocket controls the fate of the gaseous molecule during catalysis and shields the catalytic center from oxygen and water. This exquisite control makes Ecrs highly efficient carboxylases outcompeting RuBisCO, the key enzyme of photosynthesis, by an order of magnitude. Our findings define the atomic framework for the future development of CO$_2$-converting catalysts in biology and chemistry.


The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

This open access article is distributed under Creative Commons Attribution-NonCommercial-NoDerivatives License 4.0 (CC BY-NC-ND).

Data deposition: Crystallography, atomic coordinates, and structure factors have been deposited in the Protein Data Bank (PDB), http://www.rcsb.org; PDB ID code: 6OWE.

$^1$To whom correspondence may be addressed. Email: toer@mpib-marburg.mpg.de, soichi.wakatsuki@stanford.edu (for X-ray crystallography), or evvhringer@ucsd.edu (for computational correspondence).

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1901471116/-/DCSupplemental.

Published online June 26, 2019.
react with CO$_2$ in the presence of O$_2$. These features make Ecrs excellent model systems to understand the details of selective CO$_2$ binding and C-C-bond formation in proteins.

The best-studied Ecr is crotonyl-CoA carboxylase/reductase (Ccr) that catalyzes the NADPH-dependent reductive carboxylation of crotonyl-CoA into (2S)-ethylmalonyl-CoA. While Ccr does not show side reactivity with O$_2$, the enzyme catalyzes the reduction of crotonyl-CoA to butyryl-CoA as a side reaction, but only in the absence of CO$_2$ and at low catalytic efficiency (Scheme 1) (17, 18). It has been suggested that this side reaction is an evolutionary remnant of Ecrs, which are evolutionarily related to enoyl thioester reductases that catalyze the ordinary reduction of enoyl-CoA esters (6, 19). Apparently, Ecrs evolved from simple reductases into reductive carboxylases by acquiring a CO$_2$-fixation function along their evolutionary trajectory.

In a previous structural study, CinF, an Ecr from Streptomyces sp. JS360, was crystallized with NADP$^+$ and octenoyl-CoA [Protein Data Bank (PDB); PDB ID code: 4A0S (20)]. A putative CO$_2$ binding pocket was proposed to be composed of Asn77, Phe170, and Glu167, which are all highly conserved in Ecrs. It was suggested that CO$_2$ is held in position by hydrogen bonding to Asn77 and Glu167 while Phe166 would undergo hydrophobic interactions with CO$_2$. Mutation of these residues suppressed the carboxylation of octenoyl-CoA (20). However, the exact role of the individual residues directing and controlling the carboxylation reaction remains enigmatic: in particular, how the gaseous CO$_2$ molecule is aligned at the active site and how the reduction side reaction is efficiently suppressed.

Here we combine experimental biochemistry, protein crystallography, and computer simulations to define the molecular interactions of CO$_2$ during C-C-bond formation at the active site of Ecrs. Our results suggest that 4 amino acids are sufficient to convert an ordinary reductase into a highly efficient carboxylase. Together, these 4 residues anchor and lock the CO$_2$ molecule in a favorable position for the attack by the reactive enolate created during catalysis. Notably, a highly ordered water molecule plays an essential role in coordinating the CO$_2$ molecule, while the active site is otherwise effectively shielded from water to suppress the reduction side reaction. Altogether our computational and experimental studies reveal the details of selective CO$_2$ binding and C-C-bond formation in the catalytic cycle of nature’s most efficient CO$_2$-fixing enzyme.

**Results**

Crystal Structure of KsCcr with Ethylmalonyl-CoA and NADPH. We solved the structure of Ccr from Kinasatospora setae (KsCcr) co-crystallized with NADPH and soaked with ethylmalonyl-CoA, the product of the carboxylation reaction, at 1.7-Å resolution (Fig. 1 and SI Appendix, Table S1, PDB ID code: 6OWE). The active site of KsCcr shares similar features with the CinF homolog previously reported (20) [PDB ID codes: 4A0S (20), 4Y0K (21), and 4G12]. In contrast to these structures that did not capture the interaction of the protein with CO$_2$, neither as free gas or covalently bound to the acyl-CoA moiety, our structure shows densities that can be interpreted as carboxylated product (SI Appendix, Fig. S1). This enabled us to identify 4 residues that potentially interact with CO$_2$, namely Asn81, Phe170, Glu171, and His365. His365 also interacts with NADPH via hydrogen bonding (3.0 Å) to the carbamoyl group of the nicotinamide, indicating a second function of His365 in coordinating the NADPH cofactor during catalysis. Similar to previously published structures we also observed an ordered water molecule between His365 and Glu171 at a distance of 2.9 and 2.7 Å, respectively. This feature is absent in structures lacking substrate and cofactor (PDB ID codes: 3HZZ and 3KRT) suggesting a role of the water molecule in the active enzyme complex.

To test the function of these residues during catalysis, we characterized different active site variants and addressed the reaction mechanism with molecular-dynamics simulations along the
minimum free-energy path within the quantum mechanics/molecular mechanics methodology.

**Kinetic Characterization of KsCcr Wild Type.** KsCcr wild type (WT) showed an apparent turnover frequency ($k_{\text{cat}}$) of $103 \pm 3$ s$^{-1}$ which is well in line with previously reported values of $10^4$ s$^{-1}$ for the Ccr of *Rhodobacter Sphaeroides* (16). Apparent $K_M$ values were $21 \pm 2$ µM (crotonyl-CoA), $37 \pm 4$ µM (NADPH), and $90 \pm 10$ µM (CO$_2$), respectively, and substrate inhibition for crotonyl-CoA was observed at a $K_I$ of $3,650 \pm 810$ µM. Under saturating amounts of CO$_2$, the enzyme showed 100% carboxylation activity and exclusively formed (2S)-ethylmalonyl-CoA. In the absence of CO$_2$, the enzyme catalyzed the reduction of crotonyl-CoA to butyryl-CoA (Table 1). Stereochemical analysis of the butyryl-CoA in D$_2$O showed that 94 ± 2% of the deuterium label was retained (Table 1), demonstrating that the reduction side reaction took place in a stereospecific manner.

**Asn81 Anchors the CO$_2$ Molecule.** How is the CO$_2$ molecule bound in the active site? A key residue is Asn81, which defines one end of the putative CO$_2$ binding pocket. Simulations of the WT enzyme exhibited a hydrogen-bond interaction between the carboxamide NH$_2$ group of Asn81 and the CO$_2$ molecule (Fig. 2A and Movie S1). When we experimentally characterized the reaction of the N81L variant in more detail, we observed a strongly decreased carboxylation reaction, as well as accumulation of a covalent reaction intermediate, a so-called C2-ene adduct (SI Appendix, Fig. S3). C2-ene adducts are also observed in WT Ecrs when the catalytic cycle is stalled, for example when CO$_2$ is omitted from the reaction mixture. The fact that a C2-ene adduct is observed in the reaction of the N81L variant even under saturating CO$_2$ conditions suggests that the interaction of the enzyme with CO$_2$ is severely disturbed by the N81L mutation. Simulations of the N81L variant revealed that most of the catalytic residues and water molecules remain in the same position, while the CO$_2$ molecule appears increasingly disordered (Fig. 2D and Movie S2). As a consequence, the minimum energy profile from the C2-adduct to the product (2S)-ethylmalonyl-CoA of N81L becomes endothermic and shows significantly higher barriers for the C-C-bond formation step compared with the WT. This explains the reduced carboxylation activity, as well as the accumulation of the C2-ene adduct that we experimentally observed in the N81L variant (Fig. 2C and F).

Our simulations show that the active site of N81L does not become more solvent accessible, so that the reduction side reaction is still suppressed at the enzyme’s active site. But, why does N81L show 90% butyryl-CoA formation? Note that C2-ene adducts are unstable and prone to spontaneous decay into butyryl-CoA and NADP$^+$ in free solution. Accordingly, the “apparent” side reaction in N81L is nonenzymatic and caused by release of the C2-ene adduct from the active site followed by its spontaneous decay in the solvent, as described before (18, 22, 23). This hypothesis is supported by our observation that stereospecificity of butyryl-CoA formation is almost completely lost in N81L compared with the WT (Table 1). Altogether, our experimental findings are well in line with the higher calculated free-energy barriers for the carboxylation step and endothermic product formation in the N81L variant (Fig. 2F), highlighting how crucial Asn81 is for correct positioning of CO$_2$.

How is the interaction of the carboxamide group of Asn81 with CO$_2$ controlled? Analysis of the interaction network of the amino acid shows hydrogen bonding of Asn81 to 2 residues in the second shell of the active site, Thr82 and Ser119. We hypothesized that these interactions are essential in pointing the carboxamide NH$_2$ group Asn81 toward the active site to enforce its interaction with the CO$_2$ molecule. Indeed, the variants T82D and S119A showed almost full carboxylation, but at more than 50-fold reduced turnover frequency (SI Appendix, Table S3), demonstrating the importance of these secondary shell residues in increasing catalytic activity of Asn81. In summary, both simulation and experimental data suggest that Asn81 is crucial to position CO$_2$ and to establish favorable interactions of the gas molecule with the enzyme during catalysis. Absence of this residue leads to lowered carboxylation efficiency and increased formation of the labile C2-ene adduct, which is not further processed by the enzyme and leaves the active site upon which it spontaneously decays in solution.

**Phe170 Shields the Active Site from Water.** The reactive nature of the enolate that is formed during the catalytic cycle of Ccr mandates that the enolate does not get into contact with water at the active site, which would inevitably lead to its protonation and

---

**Table 1.** Apparent steady-state parameters for KsCcr and its mutants expressed as mean value ± SE

| Enzyme | Substrate | $K_{\text{M}}$ µM | $V_{\text{max}}$ µM | $k_{\text{cat}}$ s$^{-1}$ | % EMC | % Label retention$^1$
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>Crotonyl-CoA</td>
<td>21 ± 2</td>
<td>3,650 ± 810</td>
<td>103 ± 3</td>
<td>100</td>
<td>94.3 ± 1.8</td>
</tr>
<tr>
<td></td>
<td>NADPH</td>
<td>37 ± 4</td>
<td>—</td>
<td>86 ± 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CO$_2^+$</td>
<td>90 ± 10</td>
<td>—</td>
<td>78 ± 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N81L</td>
<td>Crotonyl-CoA</td>
<td>ND$^3$</td>
<td>ND</td>
<td>ND</td>
<td>19</td>
<td>58.9 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>NADPH</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F170Y</td>
<td>Crotonyl-CoA</td>
<td>10 ± 1</td>
<td>558 ± 80</td>
<td>83 ± 4</td>
<td>100</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>NADPH</td>
<td>36 ± 3</td>
<td>—</td>
<td>56 ± 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CO$_2^+$</td>
<td>150 ± 20</td>
<td>—</td>
<td>56 ± 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F170A</td>
<td>Crotonyl-CoA</td>
<td>31 ± 6</td>
<td>—</td>
<td>8.3 ± 0.4</td>
<td>17</td>
<td>87 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>NADPH</td>
<td>11 ± 0.6</td>
<td>—</td>
<td>11 ± 0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H365N</td>
<td>Crotonyl-CoA</td>
<td>29.8 ± 4.2</td>
<td>—</td>
<td>5.0 ± 0.2</td>
<td>93</td>
<td>63.3 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>NADPH</td>
<td>22 ± 2</td>
<td>—</td>
<td>8.1 ± 0.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CO$_2^+$</td>
<td>1310 ± 220</td>
<td>—</td>
<td>7.4 ± 0.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E171A</td>
<td>Crotonyl-CoA</td>
<td>500 ± 62</td>
<td>—</td>
<td>5.1 ± 0.2</td>
<td>97</td>
<td>91.4 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>NADPH</td>
<td>112 ± 8</td>
<td>—</td>
<td>6.0 ± 0.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CO$_2^+$</td>
<td>155 ± 30</td>
<td>—</td>
<td>5.1 ± 0.3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

SI Appendix, Fig. S2 shows the Michaelis–Menten graphs of the original data.

$^1$Percentage of (2S)-ethylmalonyl-CoA (EMC) over total amount of products.

$^2$Deuterium label retention at the $\alpha$-position of crotonyl-CoA expressed as mean value ± SD.

$^3$Apparent $K_{\text{M}}$ values for CO$_2$ were calculated from the HCO$_3^-$ concentration in solution at pH = 8.

$^4$Not determined due to accumulation of the covalent C2-ene adduct.
formation of the butyryl-CoA side product. A role in shielding the active site from water had been previously suggested for Phe170 (20). Simulations of an F170A variant reveal conformational changes that result in a disorganization of the active site and an increase in the number of hydration sites compared with the WT (Fig. 2; compare Fig. 2B and H, Movie S3, and SI Appendix, Supplementary Pymol and Chimera Files 1a and b and 3a and b). These changes also perturb the interaction of the CO$_2$ molecule with Asn81 (Fig. 2G).

Accordingly, the CO$_2$ molecule loses its favorable position for the reaction with the enolate so that both activation barriers are increased (Fig. 2I), which is in agreement with our experimental data (Table 1). In the F170A variant the carboxylation activity is decreased to 17% at the expense of increased reduction side reactivity. Unlike in the N81L variant, however, protonation takes place in F170A with almost WT stereospecificity (Table 1), confirming that in the F170A enzyme water is able to reach the active site and directly protonate the enolate.

Some Ecrs feature a tyrosine at position 170 instead of the phenylalanine. When we tested an F170Y variant, the enzyme showed a slightly increased substrate inhibition, but otherwise very similar kinetic parameters as the WT. Most importantly, the F170Y variant displayed full conversion of crotonyl-CoA to (2S)-ethylmalonyl-CoA in the presence of saturating amounts of CO$_2$, indicating that the presence of the hydroxyl group does not affect carboxylation activity. In summary, these experiments together with the simulations showed that the phenyl rings of phenylalanine (and tyrosine) play an important role in suppressing the reduction reaction of Ecrs by water shielding.

His365 and Glu171 Coordinate an Ordered Water Molecule Interacting with CO$_2$. Opposite of Asn81 and at the other end of the putative CO$_2$-binding pocket the residues His365 and Glu171 are located. Together, these 2 residues coordinate a water molecule. In our simulations the ordered water molecule participates in hydrogen-bonded network of 3 water molecules, which interact directly with the CO$_2$ molecule during the carboxylation step (Fig. 3). What is the exact contribution of these 2 residues to catalysis, in particular in respect to CO$_2$ binding and water accessibility?

His365 serves a dual role by also coordinating the nicotinamide ring of NADPH. To preserve interaction of residue 365 with the NADPH cofactor, but interrupt its coordination of the ordered water molecule, we generated KsCcr H365N. The H365N variant showed a 20-fold decreased activity compared with the WT enzyme but still displayed 93% of carboxylated product, even though the $K_M$ for CO$_2$ was raised by more than 1 order of magnitude. These data suggest that a defect in water coordination negatively affects C–C-bond formation activity in the H365N variant. However, this does not lead to a complete hydration of the active site. Simulations of the H365N mutant revealed broken interactions of the CO$_2$ molecule with Asn81 (Fig. 3D and Movie S4). The coordination to the ordered water molecule that bridges to Glu171 is lost, and the latter residue is rotated out of the active site disfavoring CO$_2$. 

---

**Fig. 2.** Left, Middle, and Right columns represent the WT, N81L, and F170A variants respectively. (A, D, and G) Active site of KsCcr variants before the nucleophilic attack of the enolate onto CO$_2$. An overlay of different conformations of the CO$_2$ molecule visualizes the tumbling motion in the different enzyme variants. CO-ester is shown in salmon and NADP$^+$ in cyan, and the red sphere represents the conserved water molecule coordinated by His365 and Glu171. (B, E, and H) Hydration sites located within 5 Å of the C$_\alpha$ obtained with SSTMap. For each enzyme, representative structures of the reactant state were subjected to 1 ns of constrained quantum mechanics/molecular mechanics simulations. The different positions sampled by the water molecules allowed the determination of clusters of the solvent within the active site. (C, F, and I) Minimum free-energy path from the C2-ene adduct through the enolate to the products (2S)-ethylmalonyl-CoA and NADP$^+$. Parameter D represents the distance between C$_\alpha$ and CO$_2$.
binding, which explains the experimentally observed increased $K_M$ for CO$_2$. In our simulations, the CO$_2$ molecule shows an increased rotational tumbling at the active site of the H365N variant compared with the WT, which is reflected by an increased rmsd for CO$_2$ (2.4 Å versus 0.46 Å). Together, experiment and simulations indicated reduced carboxylation efficiency because of decreased control of CO$_2$ (but not water) at the active site in the H365N variant, lowering the chances of productive Michaelis complex formation.

Similar to the H365N variant, replacement of Glu171 with alanine also resulted in a $k_{cat}$ decrease (17-fold), and carboxylation of crotonyl-CoA was also maintained (97%). Additionally, however, the $K_M$ for crotonyl-CoA increased 25-fold, indicating an additional role of Glu171 in positioning the CoA substrate. In our simulations of the E171A mutant, interaction of CO$_2$ with Asn81 is partially maintained, while Phe170 adopts the position of the mutated glutamate in the WT (Fig. 3A and Movie S5). As for H365N, the E171A variant showed increased CO$_2$ tumbling at the active site, which is reflected by an rmsd of 2.0 Å (compared with 0.46 Å of the WT). Altogether, these results suggest that in the E171A variant, similar to the H365N variant, control of the CO$_2$ molecule (but not water) is affected.

While H365N and E171A show a similar carboxylation behavior, the free-energy paths of the 2 variants show distinct differences (Fig. 3C and F). In both variants, the 2 main barriers along the minimum free-energy path of the reaction—the one that leads to the enolate from the C2-ene adduct and the one that adds CO$_2$ to the enolate—appear increased compared with the WT (for WT see Fig. 2C). This is experimentally reflected by the decreased catalytic activity of the 2 variants and can be related to a distorted water network and increased tumbling of CO$_2$ at the active site of these enzymes. In H365N, however, the first energy barrier is higher compared with E171A. This indicates that enolate formation is disfavored and suggests that the C2-ene adduct accumulates in the H365N variant. This was experimentally confirmed by measuring the stereospecificity of the reduction reaction in the absence of CO$_2$. In the H365N variant, stereospecificity was lost (Table 1), indicating that the H365N is additionally affected in enolate formation compared with the E171A mutant and the WT, respectively.

In summary, His365 and Glu171 can partially compensate each other so that carboxylation function is maintained. However, only the combined action of the 2 residues allows full control over the CO$_2$ molecule and thus a fast carboxylation rate.

### Discussion

Ccr from *K. setae* carboxylates crotonyl-CoA at a turnover frequency of more than 100 s$^{-1}$. This is almost 1 order of magnitude faster than an average RuBisCO homolog and one of the fastest CO$_2$-conversion rates described to date. Combining X-ray crystallography, experimental biochemistry, and molecular dynamics simulations we characterized the role of individual amino acids at the active site of KsCcr in CO$_2$ binding and C=C-bond formation. In KsCcr the active site is optimized to accommodate CO$_2$ and at the same time exclude water to suppress the competing reduction reaction. All this is apparently achieved by only 4 amino acids: Asn81, His365, Glu171, and Phe170. The amide group of Asn81 is responsible for anchoring the CO$_2$ from one side, while a water network organized from an ordered water molecule coordinated between His365 and Glu171 serves as an additional anchor point for CO$_2$ from the opposite side. The aromatic ring of Phe170 side chain actively prevents the diffusion of water into the active site.

Why is the CO$_2$-fixation reaction of Ecrs so much faster but still more specific compared with the reaction catalyzed by RuBisCO, although both enzymes react through an enolate? Note that there are fundamental differences in the catalytic mechanisms of RuBisCO and Ecrs. In RuBisCO, enolization of the substrate ribulose 1,5-bisphosphate is achieved by abstraction of the H3 proton through an active-site camphor-glycylated lysine, which is a reversible process and very close to equilibrium (24). (Re)protonation of the enolate yields the substrate again, which can undergo another round of activation until it reacts with a CO$_2$ (or O$_2$) molecule, which pulls the reaction further (25). In contrast, in Ecrs the enolate is formed by hydride transfer from NADPH to the β-position of crotonyl-CoA (17–19). This provides a more “unidirectional” reaction path and leaves the enolate committed for a nucleophilic attack. Accordingly, Ecrs are required to carefully control the further fate of the enolate and especially to prevent its protonation, which would irreversibly quench the reaction (in stark contrast to the case of RuBisCO). When CO$_2$ is absent from the active site of WT Ecr (18), or its positioning is disturbed (e.g., by H365N or N81L mutation, this study), the enolate is not simply transformed back into the starting substrates as in RuBisCO, but tends to collapse into the C2-ene adduct, which is in line with the idea of unidirectionality in Ecr catalysis. It might be tempting to speculate that the C2-ene adduct serves as a way to “store” the reactive enolate until a resolving CO$_2$ electrophile becomes available, thereby increasing the overall reactivity of Ecrs compared with RuBisCOs.
RuBisCO and Ecrs probably both evolved from non–CO$_2$-fixing ancestors. While it has been speculated that RuBisCO emerged from an ancestral sugar phosphate isomerase it is thought that Ecrs evolved from a primordial enoyl-CoA reductase (6, 26). Clearly, this puts more constraints onto the active-site topology of Ecrs, because unlike the CO$_2$ fixation reaction in RuBisCO that could be simply built on top of a reversible isomerization reaction, the reduction reaction needed to be suppressed and replaced by the carboxylation reaction in the Ecr scaffold because of the unidirectionality of Ecr's catalytic mechanism. The situation is reminiscent of 2-ketopropyl coenzyme M oxidoreductase/carboxylase that evolved within the superfamily of NAD[P]H disulfide oxidoreductases for which the enzyme also had to replace an active-site topology prone to reduction reactions by a CO$_2$-fixing active site (11). In both cases, the active-site transformation was achieved with only little changes and notably without introduction of a competing oxygenation reaction, posing the question whether the evolutionary circumstances or the nature of the catalytic cycle of both enzymes were responsible to achieve this superior selectivity against oxygen compared with RuBisCO.

In summary, our findings provide detailed insights into the molecular control of CO$_2$ at one of nature's most efficient carbon-fixing enzymes. While these insights will be helpful in the future design of catalytic frameworks for the capture and conversion of CO$_2$ in chemical and biology (27), but also for efforts that aim at using Ecrs as key enzymes in the development of synthetic cycles for the sustainable and efficient fixation of CO$_2$ (28, 29).

Materials and Methods

Chemicals. Crotonic Anhydride and Carbonic anhydrase from bovine erythrocytes were purchased from Sigma-Aldrich AG, CoA trilithium salt and DNase I from Roche Diagnostics, and NADPH NaN$_3$ (98%) from Carl Roth GmbH. Solvents and salts were all analytical grade or better. Crotonyl-CoA was synthesized as previously reported (30).

Cloning and Mutagenesis. The KsCcr gene was provided by the Joint Genome Institute. Enzyme variants were generated with the QuikChange Site-Directed Mutagenesis Kit (Stratagene) using primer pairs listed in SI Appendix, Table S2.

Protein Expression and Purification. Details about expression and purification are described in SI Appendix.