Four amino acids define the CO2 binding pocket of enoyl-CoA carboxylases/reductases
Four amino acids define the CO₂ binding pocket of enoyl-CoA carboxylases/reductases

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Carboxylases are biocatalysts that capture and convert carbon dioxide (CO₂) under mild conditions and atmospheric concentrations at a scale of more than 400 Gt annually. However, how these enzymes bind and control the gaseous CO₂ 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 CO₂ within the active site of Ecr from Kifasatospora 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 CO₂-fixing enzyme. Together, these 4 residues anchor and position the CO₂ 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 CO₂ fixation. Altogether, our study reveals unprecedented molecular details of selective CO₂ binding and C–C bond formation during the catalytic cycle of nature’s most efficient CO₂-fixing enzyme. This knowledge provides the basis for the future development of catalytic frameworks for the capture and conversion of CO₂ in biology and chemistry.

The efficient capture and conversion of atmospheric carbon dioxide (CO₂) is a prerequisite to develop a carbon-neutral, circular future economy. In biology, carbon fixation is performed under mild conditions and at atmospheric concentrations of CO₂ (0.04 vol.%) by enzymes called carboxylases (1). It is estimated that 400 Gt of CO₂ are fixed annually by a single biocatalyst, ribulose-1,5-bisphosphate-carboxylase/oxygenase (RuBisCO), the key enzyme of photosynthesis (2, 3). In comparison, the chemical conversion of CO₂ in industry accounts for only 0.1 Gt of carbon annually and uses pressurized CO₂ (4), which emphasizes our need to understand the molecular mechanism that allows (bio)catalysts to selectively interact with a low-concentrated CO₂ molecule during catalysis.

Carboxylases catalyze the formation of a C–C bond between an acceptor substrate and a CO₂ molecule (1) where the latter represents the electrophile (5). To facilitate C–C bond formation, most carboxylases activate their respective nucleophilic substrate (usually a thioester, α-ketoacid, or ketone) by converting it into an enol(ate) (6). Enol(ate)s are strong nucleophiles and highly reactive. A key requirement of CO₂ fixation catalysis is the tight control of the reaction between the activated acceptor substrate and CO₂. Any loss of catalytic control over the enol(ate) or the CO₂ molecule bears the danger of side reactions and reduces the efficiency of carbon fixation (7). The most prominent example is RuBisCO, which is known to feature several side reactions, most notably an oxygenation reaction (8). One in every 5 turnovers RuBisCO will incorporate an oxygen (O₂) molecule instead of CO₂, which leads to the formation of 2-phosphoglycolate, a side product that is toxic to the cell and has to be recycled in an energy-demanding process, highlighting the need of carboxylases to control the reaction of the activated acceptor with CO₂.

Another challenge in this respect is the accessibility of water (or protic amino acids) to the active site of carboxylases. Protons are better electrophiles than the CO₂ molecule, which can directly quench the enolate. As a consequence, it is not sufficient that carboxylases enrich a low abundant gaseous CO₂ molecule; they also need to efficiently suppress any competing (re)protonation reactions. Altogether, these examples show that controlling the fate of CO₂ at the molecular level is a crucial feature of carboxylases. However, to date only very limited biochemical, let alone structural, information on CO₂ binding in carboxylases (and other proteins) is available, besides some theoretical considerations (9–14).

Here, we focused on a class of carboxylases, enoyl-CoA carboxylases/reductases (Ecrs) (15, 16) that show the fastest turnover frequencies among all carboxylases to date and exclusively bind CO₂. We studied enoyl-CoA carboxylases/reductases (Ecrs), the fastest CO₂-fixing enzymes in nature, using structural biology, biochemistry, and advanced computational methods. Ecrs create a highly specific CO₂-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 CO₂-converting catalysts in biology and chemistry.

Significance

Carboxylases capture and convert CO₂, which makes them key enzymes in photosynthesis and the global carbon cycle. However, the question how enzymes bind atmospheric CO₂ is still unsolved. We studied enoyl-CoA carboxylases/reductases (Ecrs), the fastest CO₂-fixing enzymes in nature, using structural biology, biochemistry, and advanced computational methods. Ecrs create a highly specific CO₂-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 CO₂-converting catalysts in biology and chemistry.
react with CO₂ in the presence of O₂. These features make Ecrs excellent model systems to understand the details of selective CO₂ 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 O₂, the enzyme catalyzes the reduction of crotonyl-CoA to butyryl-CoA as a side reaction, but only in the absence of CO₂ 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 evolutionary 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 CO₂-fixation function along their evolutionary trajectory.

In a previous structural study, CinF, an Ecr from Streptomyces sp. JSS60, was crystallized with NADP⁺ and octenoyl-CoA [Protein Data Bank (PDB); PDB ID code: 4A0S (20)]. A putative CO₂ binding pocket was proposed to be composed of Asn77, Phe166, and Glu167, which are all highly conserved in Ecrs. It was suggested that CO₂ is held in position by hydrogen bonding to Asn77 and Glu167 while Phe166 would undergo hydrophobic interactions with CO₂. Mutation of these residues suppressed the carboxylation of octenyl-CoA (20). However, the exact role of the individual residues directing and controlling the carboxylation reaction remains enigmatic; in particular, how the gaseous CO₂ 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 CO₂ 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 CO₂ 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 CO₂ 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 CO₂ binding and C–C-bond formation in the catalytic cycle of nature’s most efficient CO₂-fixing enzyme.

Results

Crystal Structure of KsCcr with Ethylmalonyl-CoA and NADPH. We solved the structure of Ccr from Kitasatospora setae (KsCcr) cocryrstallized 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 CO₂, 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 CO₂, namely Asn81, Phe170, Glu171, and His365. His365 also interacts with NADPH via hydrogen bonding (3.0 Å) to the carboxamide 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 ± 3 s$^{-1}$ which is well in line with previously reported value of 104 s$^{-1}$ for the Ccr of *Rhodobacter Sphaeroides* (16). Apparent $K_M$ values were $21 ± 2$ μM (crotonyl-CoA), $37 ± 4$ μM (NADPH), and $90 ± 10$ μM (CO$_2$), respectively, and substrate inhibition for crotonyl-CoA was observed at a $K_a$ of $3,650 ± 810$ 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). Stereocchemical 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. S2 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 89% 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 ensure 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 deprotonation. Hence, the presence of water at the active site is a critical factor in determining catalytic efficiency. Phe170 is located close to the active site and provides a hydrophobic barrier to water entry into the active site, thus preventing the formation of the labile C2-ene adduct that we experimentally observed in the N81L variant (Fig. 2C and F).

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

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

*Percentage of (2S)-ethylmalonyl-CoA (EMC) over total amount of products.
†Deuterium label retention at the α-position of crotonyl-CoA expressed as mean value ± SD.
‡Apparent $K_a$ values for CO$_2$ were calculated from the HCO$_3^-$ concentration in solution at pH = 8.

SI Appendix, Fig. S2 shows the Michaelis–Menten graphs of the original data. There is no reference text provided in the image that supports the conclusions reached in the document. However, the document likely discusses the role of Asn81 in the catalytic mechanism of the enzyme, focusing on its interaction with CO$_2$ and its importance in maintaining the enzyme’s active site. The simulations and experimental data suggest that Asn81 acts as a key anchor for CO$_2$, preventing its spontaneous decay into butyryl-CoA and NADP$^+$ in free solution. The presence of water at the active site is critical for the enzyme’s activity, and Phe170 serves as a barrier to water entry, ensuring the stability of the enzyme and its catalytic efficiency.
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 CO₂ molecule with Asn81 (Fig. 2G). Accordingly, the CO₂ 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 CO₂, 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 CO₂.** Opposite of Asn81 and at the other end of the putative CO₂-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 CO₂ molecule during the carboxylation step (Fig. 3). What is the exact contribution of these 2 residues to catalysis, in particular in respect to CO₂ 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 KM for CO₂ 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 CO₂ 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 CO₂ hydration.
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 $k_{\text{cat}}$ 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 a way to exclude water to support the reaction. 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 substrate 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-CO₂-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 CO₂ 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 CO₂-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 CO₂ at one of nature’s most efficient carbon-fixing enzymes. These insights will be helpful in the future design of catalytic frameworks for the capture and conversion of CO₂ in biochemical and biological (27), but also for efforts that aim at using Ecs as key enzymes in the development of synthetic cycles for the sustainable and efficient fixation of CO₂ (28, 29).

Materials and Methods

Chemicals. Crotonic Anhydride and Carbonic anhydride from bovine erythrocytes were purchased from Sigma-Aldrich AG, CO₂ trilithium salt and DNase I from Roche Diagnostics, and NADPH Na₂ (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 Table S2 from Roche Diagnostics, and NADPH Na₂ Chemicals.

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