Carboxylation mechanism and stereochemistry of crotonyl-CoA carboxylase/reductase, a carboxylating enoyl-thioester reductase

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Chemo- and stereoselective reductions are important reactions in chemistry and biology, and reductases from biological sources are increasingly applied in organic synthesis. In contrast, carboxylases are used only sporadically. We recently described crotonyl-CoA carboxylase/reductase, which catalyzes the reduction of (*E*)-crotonyl-CoA to butyryl-CoA but also the reductive carboxylation of (*E*)-crotonyl-CoA to ethylmalonyl-CoA. In this study, the complete stereochemical course of both reactions was investigated in detail. The pro-(4*R*) hydrogen of NADPH is transferred in both reactions to the *re* face of the C3 position of crotonyl-CoA. In the course of the carboxylation reaction, carbon dioxide is incorporated in anti fashion at the C2 atom of crotonyl-CoA. For the reduction reaction that yields butyryl-CoA, a solvent proton is added in *anti* fashion instead of the CO_2. Amino acid sequence analysis showed that crotonyl-CoA carboxylase/reductase is a member of the medium-chain dehydrogenase/reductase superfamily and shares the same phylogenetic origin. The stereospecificity of the hydride transfer from NAD(P)H within this superfamily is highly conserved, although the substrates and reduction reactions catalyzed by its individual representatives differ quite considerably. Our findings led to a reassessment of the stereospecificity of enoyl(-thioester) reductases and related enzymes with respect to their amino acid sequence, revealing a general pattern of stereospecificity that allows the prediction of the stereochemistry of the hydride transfer for enoyl reductases of unknown specificity. Further considerations on the reaction mechanism indicated that crotonyl-CoA carboxylase/reductase may have evolved from enoyl-CoA reductases. This may be useful for protein engineering of enoyl reductases and their application in biocatalysis.

alcohol dehydrogenase | biocatalysis | enoyl reductase

The use of enzymes in organic chemistry has been increasing steadily in recent years, because enzymatic catalysis provides some advantages over classical synthesis methods. Besides the capability to promote reactions under mild conditions, the high regio- and stereoselectivity of biocatalysts has received much attention (1). Reductases represent an important class of enzymes that are used in organic synthesis, with alcohol dehydrogenases and enoate reductases as the most prominent examples. Enoate reductases are unique in their ability to reduce selectively C═C bonds in α,β-unsaturated carbonyl compounds and to create thereby up to 2 stereogenic centers in the target molecule. This chemo- and stereoselectivity makes enoate reductases an important addition to the synthetic toolbox (1–4).

Another challenge in organic synthesis is the introduction of carboxyl groups into a target molecule (5). Direct carboxylations of organic substrates are poorly represented in organic synthesis, and the few examples, such as the Kolbe–Schmitt reaction or the Grignard reaction require quite harsh conditions (Kolbe–Schmitt) or an inert atmosphere and nonaqueous solvents (Grignard) (6). Although carboxylation reactions occur widely in nature, carboxylating enzymes are rarely used in organic synthesis. The only prominent examples reported thus far are phenylphosphate carboxylase (6) and pyrrole-2-carboxylate decarboxylase (7). The application of most carboxylases is limited, because many carboxylating enzymes require either complex or unstable substrates (e.g., ribulose-1,5-bisphosphate, phosphorenolpyruvate), depend on cofactors (e.g., ATP, biotin), metal ions, or are multienzyme complexes difficult to prepare for synthetic purposes [supporting information (SI) Table S1].

We recently reported the discovery of an enzyme, crotonyl-CoA carboxylase/reductase (Ccr) from *Rhodobacter sphaeroides*, that represents a type of carboxylase and catalyzes the reductive carboxylation of (*E*)-crotonyl-CoA to ethylmalonyl-CoA with reduced nicotinamide adenine dinucleotide phosphate (NADPH) as reductant (8). Acryloyl-CoA is accepted as an alternative substrate analogue by the enzyme with 40% relative activity (compared with v_{max} of crotonyl-CoA carboxylation), yielding methylmalonyl-CoA. Interestingly, crotonyl-CoA carboxylase/reductase is related to dehydrogenases that reduce C═C or C═O bonds, and Ccr also catalyzes the reduction of (*E*)-crotonyl-CoA to butyryl-CoA in the absence of HCO_3^-/CO_2, albeit with only 10% of maximal activity (compared with v_{max} of crotonyl-CoA carboxylation), indicating that the carboxylation reaction is the physiologically relevant reaction (Fig. 1*A*). The properties of Ccr are summarized in Table 1.

In this work, the carboxylation mechanism and the stereochemical course of both the carboxylation reaction and the reduction reaction of Ccr were investigated in detail. The results reported herein led to a reassessment of the stereochemical diversity with respect to the amino acid sequence diversity of enoyl (-thioester) reductases and revealed interesting aspects about the evolution of these enzymes, with possible implications for protein engineering and the use of those enzymes in biocatalysis.

Results

Mechanism of Carboxylation (Carboxylating Species). To investigate the mechanism of carboxylation, the oxidation of NADPH in a mixture of crotonyl-CoA* and Ccr was followed spectrophotometrically at 360 nm upon the addition of either CO_2 or HCO_3^-. In principle, either CO_2 or HCO_3^-, can serve as active species of CO_2 in enzymatic carboxylation reactions (9). Because the hydration of CO_2 ("dissolved" $CO_2 + H_2O$ D H_2CO_3 D $HCO_3^- + H^+$) is slow at temperatures <20 °C, a difference in the initial enzymatic rate can be observed, when nonsaturating concentrations of either dissolved CO_2 or HCO_3^- are added to start the reaction at low

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*From here on, "crotonyl-CoA" is used for (*E*)-crotonyl-CoA.

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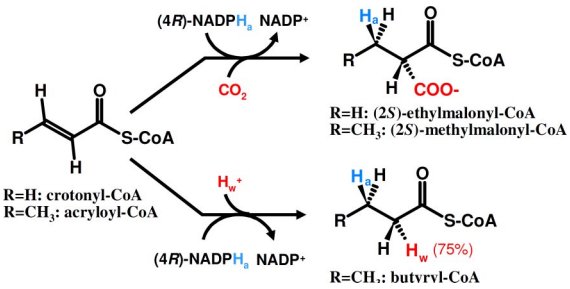

Fig. 1. Crotonyl-CoA carboxylase/reductase. Reactions catalyzed by Ccr. Properties of crotonyl-CoA carboxylase/reductase are shown in Table 1.

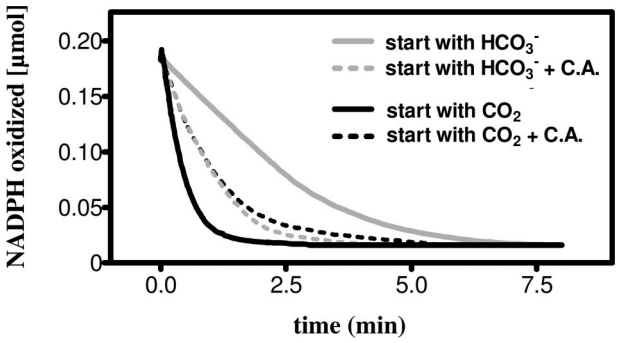

Fig. 2. Determination of the carboxylating species of the Ccr reaction. The oxidation of NADPH during the reductive carboxylation of crotonyl-CoA to ethylmalonyl-CoA was followed spectrophotometrically at 360 nm. To determine the carboxylating species, the reaction was started either with dissolved CO_2 (black solid line), or HCO_3^- (gray solid line) at 15 °C, a temperature, at which the hydration of CO_2/dehydration of HCO_3^- is slow. As control, the reaction was started also with dissolved CO_2 (black dotted line) or HCO_3^- (gray dotted line) in the presence of carbonic anhydrase (C.A.), an enzyme that catalyzes the reversible hydration of CO_2.

temperatures, depending on which species is used as the substrate (9, 10). In the case of Ccr, the oxidation of NADPH that corresponds to the formation of ethylmalonyl-CoA was faster when the reaction was started with dissolved CO_2 compared with HCO_3^-, strongly indicating that CO_2 is the carboxylating species in this reaction (Fig. 2). Addition of carbonic anhydrase to the reaction mixture led to identical reaction rates, independent of the CO_2 species that was used in the reaction mixture. Because carbonic anhydrase catalyzes the reversible hydration of CO_2 and therefore strongly increases the velocity of equilibration between dissolved CO_2 and HCO_3^- (11) that is limited under these conditions, these results support the contention that CO_2 is the reactive species.

Stereochemistry of the Carboxylation Products. The stereochemistry of the carboxyl group that is introduced at the C2 atom was elucidated by enzymatic analysis of methylmalonyl-CoA as the product of acryloyl-CoA carboxylation by Ccr. Incubation of Ccr with acryloyl-CoA, NADPH and ^{14}C-labeled $NaHCO_3$ yielded [3-carboxy-^{14}C]-methylmalonyl-CoA (Fig. 3*A*) that was incubated subsequently with methylmalonyl-CoA epimerase (Epi) and/or (2*R*)-methylmalonyl-CoA mutase (Mcm). Methylmalonyl-CoA epimerase catalyzes the isomerization of (2*S*)-methylmalonyl-CoA and (2*R*)-methylmalonyl-CoA, whereas (2*R*)-methylmalonyl-CoA mutase catalyzes the coenzyme B_{12}-dependent carbon skeleton rearrangement of (2*R*)-methylmalonyl-CoA to succinyl-CoA (Fig. S1) (12, 13). HPLC analysis showed succinyl-CoA formation only in the presence of both enzymes (Epi and Mcm), whereas incubation with either Epi or Mcm alone did not result in formation of succinyl-CoA (Fig. 3 *B–D*). We therefore conclude that carboxylation proceeds to the *re* face at the C2 atom (Fig. S2), resulting in products with (2*S*)-stereochemistry.

Stereochemistry of the Hydride Transfer (Carboxylase Reaction). The hydride that is transferred from NADPH onto a substrate can be derived either from the pro-(4*S*) or pro-(4*R*) position at C4 of the nicotinamide ring (Fig. S2). To investigate the stereochemistry of this transfer, [^{2}H]-(4*R*)- and [^{2}H]-(4*S*)-NADPH were synthesized enzymatically and purified. The position of label was confirmed by NMR (see Fig. S3), and the labeled coenzymes were used in subsequent experiments. Incubation of crotonyl-CoA (m/z = 834) in the presence of HCO_3^- and NADPH or [^{2}H]-(4*S*)-NADPH yielded ethylmalonyl-CoA with m/z = 880 as identified by HPLC-MS (Fig. 4 *A* and *B*). Conversely, incubation of crotonyl-CoA with [^{2}H]-(4*R*)-NADPH resulted in ethylmalonyl-CoA of m/z = 881, indicating that a ^{2}H-transfer had taken place (Fig. 4C). This demonstrated that the reductive carboxylation reaction of Ccr is pro-(4*R*) specific with respect to NADPH.

Stereochemistry of the Hydride Transfer (Reductase Reaction). The stereochemistry of the hydride transfer from NADPH was also investigated for the reduction reaction of Ccr that takes place in the absence of HCO_3^-/CO_2. Incubation of crotonyl-CoA with Ccr resulted under these conditions in butyryl-CoA of m/z = 836, when unlabeled NADPH (Fig. 5*A*) or [^{2}H]-(4*S*)-NADPH was used, as shown by HPLC-MS. [^{2}H]-(4*R*)-NADPH yielded butyryl-CoA with m/z = 837 (Fig. 5*B*), demonstrating that the reduction reaction of Ccr is pro-(4*R*) specific. The stereochemistry of the hydride transfer is thus conserved for both reactions catalyzed by Ccr.

Cryptic Stereochemistry at the C3 Atom of the Product (Reductase Reaction). To investigate the stereochemistry of the hydride that is transferred to the C3 atom of the product, butyryl-CoA (labeled or unlabeled) was isolated from the reaction mixtures described above by preparative HPLC. These [^{2}H]-C3-labeled butyryl-CoA and

Table 1. Properties of crotonyl-CoA carboxylase/reductase

Parameter	Recombinant crotonyl-CoA carboxylase/reductase
Reductive carboxylation	
Specific activity (V_{max})	40 units[§] mg^{-1} (this study, tagged enzyme 100 units mg^{-1}
Substrates	Crotonyl-CoA (100% relative specific activity),acryloyl-CoA (40%)
	Crotonyl-*N*-acetylcysteamine, methacryloyl-CoA, 6-hydroxycylohex-1-ene-1-carboxyl-CoA, cyclo-hexa-1,5-diene-1-carboxyl-CoA, acetoacetyl-CoA, propionyl-CoA, (*R*)-3-hydroxybutyryl-CoA, (*S*)-3-hydroxybutyryl-CoA (all <1%)
Apparent K_m values	0.4 mM crotonyl-CoA (0.5 mM acryloyl-CoA); 0.7 mM NADPH; 14 mM HCO_3^- (equivalent to 0.2 mM "dissolved CO_2" at pH 7.8)
pH optimum	7.5–8.0
Molecular composition	Native molecular mass: 105 + 11 kDa, subunit molecular mass: 47 kDa
Reduction	
Specific activity	3 units mg^{-1} (this study, tagged enzyme) 10 units mg^{-1} (according to ref. 8, nontagged enzyme)
Apparent K_m values	0.2 mM crotonyl-CoA; NADPH not determined

[§]One unit corresponds to 1 mmol of product formed per min.

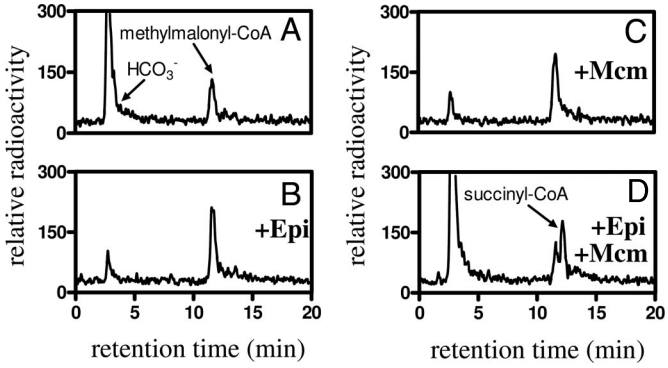

Fig. 3. Determination of the stereochemistry of the carboxylation product. (*A*) Acryloyl-CoA was incubated with NADPH, Ccr, and ^{14}C-labeled $NaHCO_3$, resulting in radioactive-labeled methylmalonyl-CoA as shown by HPLC and radioactive monitoring. (*B–D*) To determine the stereochemistry of the product, this methylmalonyl-CoA was subsequently incubated for 1 min with methylmalonyl-CoA epimerase (Epi) (*B*), (2*R*)-methylmalonyl-CoA mutase (Mcm) (*C*), or a combination of both enzymes (*D*). The formation of radioactive labeled products was followed by HPLC.

unlabeled butyryl-CoA species were incubated with butyryl-CoA dehydrogenase. This flavoenzyme stereospecifically removes the pro-(2*R*)- and pro-(3*R*)-hydrogen atoms of butyryl-CoA yielding crotonyl-CoA (14). In all cases, the products formed (crotonyl-CoA) had a *m/z* of 834, according to a loss of label during the oxidation of [^{2}H]-C3 labeled butyryl-CoA, as determined by HPLC-MS (Fig. 5 *A* and *B*). This indicates that the hydride transferred from NADPH onto the C3 of butyryl-CoA occurs to the *re* face (Fig. S2) resulting in pro-(3*R*)-butyryl-CoA.

Determination of the Stereospecificity of the Solvent Proton Addition (Reductase Reaction). To determine the stereochemistry of the solvent proton that is added at the C2 position of crotonyl-CoA, the reduction of crotonyl-CoA to butyryl-CoA was performed in $[^2H]_2O$ in the absence of HCO_3^-/CO_2. Incubation of crotonyl-CoA

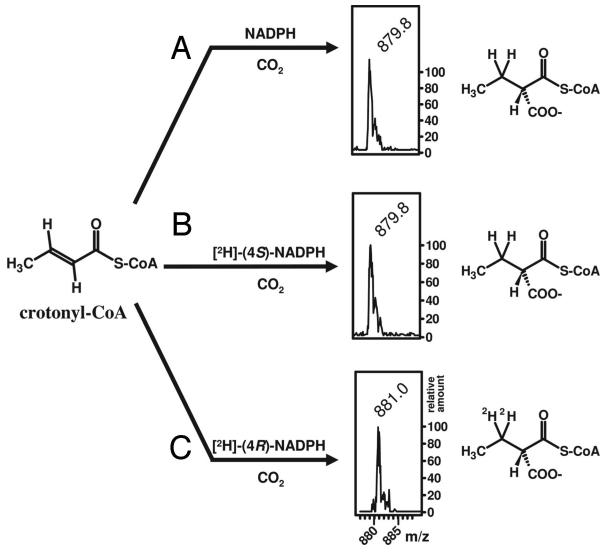

Fig. 4. Determination of the stereochemistry of the hydride transfer (carboxylase reaction). The stereospecificity of the hydride transfer from the prochiral C4 position of the nicotinamide was determined by using stereospecifically labeled NADPH. Crotonyl-CoA was incubated in the presence of HCO_3^-/CO_2 with unlabeled NADPH (*A*), [^{2}H]-(4*S*)-NADPH (*B*), or [^{2}H]-(4*R*)-NADPH (*C*). The products were analyzed by HPLC-MS. The corresponding mass spectra of the ethylmalonyl-CoA species formed are shown in detail.

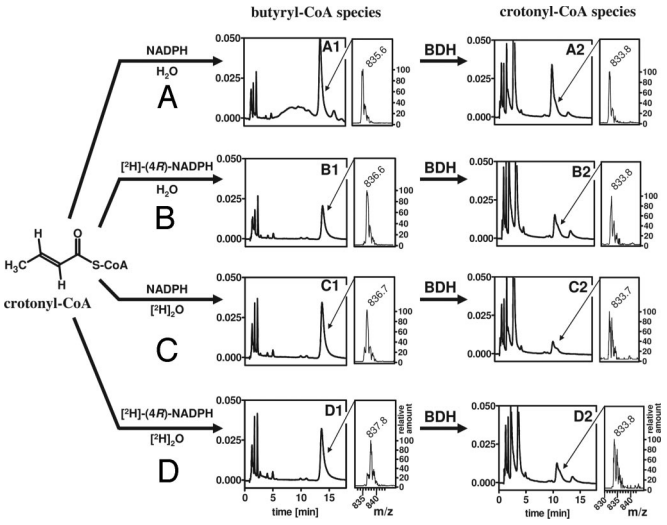

Fig. 5. Determination of the stereochemistry of the reductase reaction. (*A* and *B*) Stereochemistry at C3. Crotonyl-CoA was incubated in the absence of HCO_3^-/CO_2 with Ccr and either NADPH or [^{2}H]-(4*R*)-NADPH, and the butyryl-CoA species formed were analyzed by HPLC-MS. The corresponding HPLC chromatograms (*A1*, *B1*) are shown together with the detailed mass spectra of the respective butyryl-CoA peaks. The butyryl-CoA species were isolated by preparative HPLC from the reaction mixture and converted back to crotonyl-CoA by pro-(2*R*), pro-(3*R*)-specific butyryl-CoA dehydrogenase (BDH) from pig liver mitochondria, to determine the absolute stereochemistry of the label incorporated. The corresponding HPLC chromatograms (*A2*, *B2*) and the respective mass spectra of crotonyl-CoA species are shown. (*C* and *D*) Stereochemistry at *C2*. Crotonyl-CoA was incubated in the absence of HCO_3^-/CO_2 in $[^2H]_2O$ with Ccr and either unlabeled NADPH or [^{2}H]-(4*R*)-NADPH and the butyryl-CoA species formed were analyzed by HPLC-MS. The corresponding HPLC chromatograms (*C1*, *D1*) and detailed mass spectra of the butyryl-CoA peaks are shown. After isolation of the butyryl-CoA species from the reaction mixtures by preparative HPLC, the CoA-esters were converted back to crotonyl-CoA by butyryl-CoA dehydrogenase (BDH) to determine the absolute stereochemistry of the label incorporated. The HPLC chromatograms (*C2*, *D2*) and mass spectra of crotonyl-CoA species are shown in detail.

with NADPH in $[^2H]_2O$ yielded butyryl-CoA at a predominant *m/z* of 837, indicating that a ^{2}H of the solvent had been incorporated (Fig. 5*C*). HPLC-MS also showed that ≈10% of butyryl-CoA with a *m/z* of 836 were present. This corresponds exactly to the amount of unlabeled H_2O (10% vol/vol) that is brought into the reaction mixture by the aqueous Ccr solution. Consequently, incubation of crotonyl-CoA with [^{2}H]-(4*R*)-NADPH in $[^2H]_2O$ resulted in butyryl-CoA with *m/z* = 838 and ≈10% of *m/z* = 837 (Fig. 5*D*). [^{2}H]-C2- and [$^2H_2$]-C2,C3-double-labeled butyryl-CoA that had been isolated by preparative HPLC were both transformed by butyryl-CoA dehydrogenase to crotonyl-CoA with a predominant *m/z* = 834 (Fig. 5 *C* and *D*), which means that in both cases, the deuterium atoms had been lost. Because butyryl-CoA dehydrogenase specifically removes the pro-(3*R*)- and pro-(2*R*)-hydrogen atoms of butyryl-CoA, these results clearly show that incorporation of the solvent hydrogen occurs predominantly at C2 to the *re* face (Fig. S2) and therefore *anti* to the hydride from NADPH that is transferred onto C3. Interestingly, when butyryl-CoA was labeled at the C2 atom, part of the label (≈25%) was not removed by butyryl-CoA dehydrogenase as expected for an enzymatic reaction. This indicates that the solvent proton is not incorporated 100% in the pro-(2*R*) position but that to a smaller extent, incorporation also occurs to the *si* face. Such a finding is not surprising considering the reductase reaction as a physiological nonrelevant "side reaction" of the enzyme when compared with the carboxylase reaction that is catalyzed 10 times faster by Ccr. The observed loss of stereocontrol in case of the reduction reaction may be due to the geometry of the active site that has evolved to direct the incorporation of a carboxyl

group that is electronically and sterically different from a (solvent) proton.

Discussion

In this study, the reactions catalyzed by crotonyl-CoA carboxylase/reductase have been investigated in detail, and the complete stereochemical course of both reactions has been elucidated. The reduction of crotonyl-CoA to butyryl-CoA occurs from pro-(4*R*)-NADPH onto the *re* face of the C3 atom of crotonyl-CoA, and the solvent proton is added mainly in *anti* fashion to the *re* face at C2 (Scheme 1). Correspondingly, in case of the reductive carboxylation of crotonyl-CoA to ethylmalonyl-CoA, the carboxyl group is also incorporated to the *re* face, resulting in products with (2*S*)-stereochemistry (Fig. S2).

Functional and Historical Models of Stereospecificity in Reduction Reactions. The stereochemistry of NAD(P)H dependent reductases/dehydrogenases has been investigated in detail for >50 years (15), and it has been shown in very early studies on >100 dehydrogenases that approximately half of the enzymes catalyze a transfer of the pro-(4*R*) hydrogen and the other half transfer the pro-(4*S*)-hydrogen (16). Moreover, crystal structures have shown that pro-(4*R*)-specific dehydrogenases bind the NAD(P)H cofactor in *anti* conformation, whereas binding of the cofactor in pro-(4*S*)-specific dehydrogenases occurs in syn conformation (see Fig. S2) (17, 18). Ab initio molecular orbital calculations agree that for the *anti* conformation of NAD(P)H, the transfer of the pro-(4*R*) hydrogen is preferred, whereas the pro-(4*S*) hydrogen is transferred preferentially if the NAD(P)H is bound in syn conformation (18), which in each case corresponds to a transfer of the hydride ion from the pseudoaxial position. It has been argued further, that NAD(P)H bound in *anti* conformation is a weaker reducing agent than NADPH bound in syn and that optimal enzymes bind substrates so as to match the free energies of the bound intermediates (16). Because of striking correlations of the stereospecificity of reductases/dehydrogenases and the change in free energy between their corresponding substrates and products, a functional model has been proposed in which the nature of the stereospecificity [pro-(4*R*) or pro-(4*S*)] is dictated by a mechanistic imperative (16). However, some exceptions have been reported in which enzymes that accept the same substrates show different stereospecificities (19), and these findings are difficult to explain by a functional model. Thus, recent analyses that take amino acid sequences into account favor a historical model, in which stereospecificity is a nonselected trait that is conserved during divergent evolution (19–21). According to this model, enzymes within a specific class that are related on the amino acid sequence level all catalyze their respective reactions with the same stereospecificity, whereas enzymes that are nonhomologous, and thus differ in their amino acid sequence, may catalyze reactions with opposite stereochemistry (20).

Enoyl (-thioester) reductases display an interesting case in which both a functional and a historical model are considered to explain the stereochemistry observed. In all cases examined, the addition of the solvent proton occurs at C2. This regioselectivity is consistent with a functional model due to the strong polarization of the α–β unsaturated double bond (20). In contrast, the observed diversity in stereoselectivity of the addition at C2 and C3 has been correlated to distinct amino acid sequences that reflect different evolutionary origins of the respective enzymes (20).

Stereospecificity of Ccr and Related Reductases/Dehydrogenases. Ccr is distantly related to proteins of the medium-chain dehydrogenase/reductase superfamily, among which are alcohol dehydrogenase from horse liver (amino acid sequence identity/similarity: 22/36%) (22), ζ-crystallin from human lens (23/39%) (23), and quinone oxidoreductase from *Escherichia coli* (24/40%) (24), as well as *Thermus thermophilus* HB8 (24/39%) (25). The crystal structures of all these proteins have shown that binding of the NAD(P)H cofactor occurs in *anti* conformation with several interactions of the pro-(4*S*) face of NAD(P)H to amino acids that are responsible for the binding of the nicotinamide, but not involved in catalysis. These steric interactions are in line with a transfer of the pro-(4*R*) hydride from NAD(P)H onto the respective substrate. In the case of horse liver alcohol dehydrogenase the pro-(4*R*) specificity of the hydride transfer has been experimentally verified (26). Altogether, these findings are well in line with the historical model, in which enzymes of a common origin and therefore of sequence similarity are supposed to catalyze reactions with the same stereospecificity, even though their substrates or the reduction reactions catalyzed (C═O reduction, C═C reduction, or reductive carboxylation) may differ quite considerably.

A crotonyl-CoA reductase that is highly similar to Ccr from *R. sphaeroides* has been isolated from *Streptomyces collinus* (amino acid identity/similarity 41/56%), and its stereochemistry was studied in detail (27). The stereochemical course of hydride transfer and proton addition at crotonyl-CoA of the *S. collinus* enzyme corresponds to that defined for Ccr. By contrast, the hydride transfer that is pro-(4*R*) specific in case of Ccr was reported to be pro-(4*S*) specific for the enzyme from *S. collinus*. This is especially notable because of the high sequence similarity of the *S. collinus* enzyme and Ccr, which suggests that the *S. collinus* enzyme may represent a bona fide crotonyl-CoA carboxylase/reductase. This is further substantiated by the fact that reductive crotonyl-CoA carboxylation occurs in *Streptomyces coelicolor* A3 (2), a close relative of *S. collinus* (8), and that this activity is catalyzed by an enzyme of 93% amino acid sequence identity to the *S. collinus* enzyme (55). An explanation may be found in the original authors' use of glucose dehydrogenase from *Thermoplasma acidophilum* to synthesize radioactive labeled (4*S*)-NADPH for the investigation of hydrogen transfer stereospecificity (27). Glucose dehydrogenase from *T. acidophilum* has since been demonstrated to be pro-(4*R*) specific, rather than pro-(4*S*) specific as originally assumed (28). Reevaluation of the original data therefore clearly shows that the *S. collinus* enzyme is actually pro-(4*R*) specific. This result is in good agreement with the conserved stereochemistry of the hydride transfer from the pro-(4*R*) position of NADPH that is observed within the superfamily of medium-chain dehydrogenases/reductases that includes Ccr and the *S. collinus* enzyme, a fact that had been overlooked for the latter one thus far (20, 27). Interestingly, the enoyl thioester reductase domains of rat liver and chicken fatty acid synthases that have been reported to be pro-(4*R*) specific are also related to the same medium-chain dehydrogenase/reductase family (29, 30). The *S. collinus* enzyme and Ccr, which share the same nucleotide cofactor stereospecificity, may therefore also be related on evolutionary grounds to those enoyl reductase domains and may not have a different evolutionary origin as initially proposed (20, 27).

General Pattern of Stereospecificity for Enoyl-(Thioester) Reductases (ER). Analysis of ERs, whose stereochemistry has been studied and the amino acid sequence is available, shows a clear correlation of cofactor stereospecificity and domain architecture (Table S2, Fig. S4). Those ERs belonging to the family of medium-chain dehydrogenases/reductases (31) contain domains of the ADH_zinc_N superfamily (pfam00107) and are all pro-(4*R*) specific. Examples are ER domains of type I fatty acid synthase from rat liver and chicken (26), the type II fatty acid synthase ER Etr1p from the yeast *Candida tropicalis* (32), or alkenal/one oxidoreductase from rat (33). By contrast, the pro-(4*S*)-specific enzymes either contain domains of the AdoHcyase superfamily (cl09931) like FabI and InhA (both type II fatty acid synthase) from *E. coli* (26) and *Mycobacterium tuberculosis* (34), or of the TIM_phosphate_binding superfamily (cl09108) like the ER domains of type I fatty acid synthase from baker's yeast (26) or 2,4-dienoyl-CoA reductase from *E. coli* (35). These correlations can be extended to the alcohol dehydrogenases of the medium-chain dehydrogenase/reductase family (horse liver, yeast and *Pseudomonas fluorescens*) that are all

pro-(4*R*) specific and harbor an ADH_zinc_N superfamily domain. Similarly, *Drosophila* (short chain) alcohol dehydrogenase and the keto-acyl carrier protein reductase Sco1815 (type II polyketide synthase, PKS) involved in R1128 biosynthesis from *S. coelicolor* (36) are both pro-(4*S*) specific and possess a domain of the AdoHcyase superfamily. For alcohol (polyol) dehydrogenases, such correlations have actually been anticipated (37), and our results on ER therefore strongly support the assumption that the cofactor stereospecificity is preserved within structural defined enzymatic domains.

When ERs of unknown stereoselectivities are analyzed, their stereochemistry can be assigned according to Table S2 (see also Fig. S4), if they contain 1 of the 3 domains described above. We therefore propose that ER domains of the type I PKS are pro-(4*R*) specific, as deduced for the respective domains of the Nystatin and Epothilone biosynthesis modules. In keeping with this, the ER LovC that acts in *trans*[†] on the (iterative) type I lovastatin PKS (38) also displays pro-(4*R*) stereospecificity, whereas Rv2953 that similarly operates in *trans* during the biosynthesis of (phenol)glycolipids in *M. tuberculosis* (39) cannot be assigned on the basis of its domain structure. However, further sequence–distance relationship analysis indicate that Rv2953 may be pro-(4*S*) specific (Fig. S4). ERs like FabK (40), FabL (41), or FabV (42) that replace the widely distributed FabI component of fatty acid synthases (type II) in a number of bacteria, most likely are pro-(4*S*) specific according to Table S2. It is noteworthy to mention that, to date, any experimental evidence for the stereospecificity of PKS ER (domains) is missing (43). This is also the case for ERs that substitute FabI in bacterial fatty acid synthesis. Clearly, further experimentation is required to prove the suggested correlation of cofactor stereospecificity and domain architecture of ERs. However, a similar correlation between substrate stereospecificity and amino acid sequence cannot be drawn easily, because the substrate that is bound to the active site as well as the reactions that are catalyzed differ quite remarkably. Therefore, the prediction of the complete stereochemistry of an enzymatic reaction by analysis of the amino acid sequence or the domain structure remains challenging.

Possible Reaction Mechanism for Ccr. Investigation of the carboxylation reaction of Ccr showed that CO_2 and not HCO_3^- is the carboxylating species. In principle, CO_2 can serve as electrophile in C—C forming reactions on negatively polarized (or inverted) carbon atoms (5), whereas HCO_3^- is a nucleophile that has to be transformed into the carboxylating species by formation of a reactive carboxyphosphate upon ATP/phosphoester hydrolysis or as carboxyl-biotin to serve in enzymatic carboxylation reactions (44, 45). Because the carboxylation reaction of Ccr is neither ATP- nor biotin-dependent (8), this further supports the role of CO_2 as a carboxylating species. Dissection of the reductive carboxylation into 2 separate "half reactions" shows that reduction of crotonyl-CoA to butyryl-CoA is an exergonic process, and that the free-energy change associated with this reduction should be able to drive the endergonic carboxylation reaction (46). Considering the experimental results, we therefore propose the following reaction mechanism for crotonyl-CoA carboxylase/reductase.[‡] First, a hydride ion is transferred from NADPH onto C3 of crotonyl-CoA to give a thioester enol(ate), followed by an electrophilic attack of the CO_2 at C2. These chemical steps may occur either simultaneously, as in a concerted mechanism, or successively, as in a stepwise mechanism. This cannot be differentiated by the current data. A small isotopic effect of ≈1.7 on the k_{cat} of the carboxylation reaction was observed when [^{2}H]-(4*R*)-NADPH was used instead of unlabeled NADPH. This suggests that the hydride transfer onto the substrate is partially involved in the rate-limiting step of the reaction (47).

A stepwise mechanism has been demonstrated for 2,4-dienoyl-CoA reductase from rat liver mitochondria that catalyzes the NADPH-dependent reduction of 2,4-dienoyl-CoA thioesters to the resulting *trans*-3-enoyl-CoA products. The formation of a dienolate anion intermediate during the course of reaction has been shown by kinetic and spectrophotometric methods (48). Similarly, incubation of Ccr with crotonyl-CoA in the absence of CO_2 may also result in the formation of such an enolate anion, because the true electrophile CO_2 is missing, and the rate-limiting step is shifted to the addition of a solvent proton replacing that CO_2 molecule. This speculation may be supported by the observation that, in contrast to the carboxylation reaction, no isotopic effect on the k_{cat} of the reduction reaction was observed when [^{2}H]-(4*R*)-NADPH was used instead of unlabeled NADPH.

Concluding Remarks. The results of this study suggest a detailed mechanistic model of the reactions catalyzed by Ccr with implications on the evolution of this enzyme class. Comparison of amino acid sequences of Ccr and well-studied members of the medium-chain dehydrogenase/reductase superfamily indicate that all enzymes share the same origin (49). One might therefore speculate that crotonyl-CoA carboxylase/reductase has emerged from reductases and that it has evolved an active center that clearly prefers the carboxylation of crotonyl-CoA over the reduction reaction. From this point of view, the reduction reaction of Ccr may be taken as an evolutionary relict, resulting in a much lower catalytic efficiency as well as less conserved stereochemistry at C2 compared with the carboxylation. In summary, the evolution of a primordial enoyl-CoA reductase toward a true carboxylase may provide the basis for further protein engineering of enoate reductases. Identification of those residues and/or structural properties that direct the carboxylation reaction and are involved in the preferential binding of a CO_2 molecule at the active site may in turn allow a rational design of enoate carboxylases (or enzymes that incorporate other electrophiles) on the scaffold of already known enoate reductases and would extend the synthetic toolbox of organic chemists.

Materials and Methods

Preparation of Enzymes and Substrates. Recombinant Ccr was produced in 200-L scale and purified from cell extracts (15 mL, 1.6 g of protein) as described (8). Epi and Mcm were prepared as described previously (50). A histidine-tagged version of Ccr(Ccr$_{his}$) was produced as described in detail in *SI Methods*. Butyryl-CoA dehydrogenase was prepared from pig liver as described (51, 52) with minor modifications (see *SI Methods*). Crotonyl-CoA and acryloyl-CoA were synthesized as reported before (50). [^{2}H]-(4*R*)- and [2H]-(4*S*)-NADPH were synthesized and purified according to Pollock and Barber (53) with some modifications (see *SI Methods* for details). The purified products were characterized by NMR (Fig. S3).

Determination of the Carboxylating Species. The active species of CO_2 was determined spectrophotometrically after a modified method (9) in a cuvette (d = 0.1 cm) at 15 °C, following the rate of NADPH oxidation at 360 nm (ε_{NADPH} = 3.4 mM^{-1} cm^{-1}). For experimental details, see *SI Methods*.

Stereochemistry of the Carboxylation Product. Radioactive-labeled methylmalonyl-CoA was synthesized from acryloyl-CoA and $H^{14}CO_3$ by Ccr and subsequently used as substrate for different combinations of Epi and/or Mcm as described recently (50). Samples were withdrawn from the reaction mixtures and analyzed subsequently by HPLC and radioactive monitoring (see below). Further details are described in *SI Methods*.

Stereospecificity of the Hydrogen Transfer from NADPH (Carboxylase Reaction). Ethylmalonyl-CoA species were synthesized from crotonyl-CoA and $NaHCO_3$ by Ccr as described before (8) by using [^{2}H]-(4*R*)-NADPH, [^{2}H]-(4*S*)-NADPH or unlabeled NADPH, respectively. The ethylmalonyl-CoA formed was analyzed by HPLC-MS (see below, *SI Methods*).

[†]"*Trans*-acting" ERs are accessory enzymes that interact with large multidomain enzymes (e.g. type I PKS) to complement the activity of a missing (or inactive) ER domain.

[‡]This model is based on a proposal from W. Buckel at the combined seminar of the Graduiertenkolleg "Protein function on atomic level" (Marburg, Germany) and "Biochemistry of the enzymes" (Freiburg, Germany) 2007 in Hirschegg, Austria.

Stereospecificity of the Hydrogen Transfer from NADPH (Reductase Reaction). Butyryl-CoA species were synthesized from crotonyl-CoA by Ccr as described above, by using [^{2}H]-(4*R*)-NADPH, [^{2}H]-(4*S*)-NADPH, or unlabeled NADPH and omitting $NaHCO_3$ from the reaction mixture. The butyryl-CoA formed was analyzed by HPLC-MS (see below, *SI Methods*).

Cryptic Stereochemistry at C3 (Reductase Reaction). [^{2}H]-C_3-butyryl-CoA (synthesized from [^{2}H]-(4*R*)-NADPH) and butyryl-CoA (synthesized from NADPH) were purified by preparative HPLC and incubated with butyryl-CoA dehydrogenase preparations in a modified assay (54) by using ferricenium hexafluorophosphate as electron acceptor. The crotonyl-CoA formed was analyzed by HPLC-MS (see below, *SI Methods*).

Stereoselectivity of the Solvent Hydrogen Addition (Reductase Reaction). Reduction of crotonyl-CoA by Ccr was performed as described by using [^{2}H]$_2$O as solvent. [^{2}H]-C_2-butyryl-CoA (synthesized from NADPH in [^{2}H]$_2$O) and [^{2}H$_2$]-C2,C3-butyryl-CoA (synthesized from [^{2}H]-(4*R*)-NADPH in [^{2}H]$_2$O) were isolated by preparative HPLC and subjected to butyryl-CoA dehydrogenase as described above.

Miscellaneous. All CoA esters were analyzed by reversed-phase HPLC(-MS) by using UV detection and radioactive monitoring as described recently (8, 50). For details, see *SI Methods*. Accession numbers and amino acid sequences of the proteins analyzed in this study are in Tables S3 and S4, respectively.

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