Cell. Mol. Life Sci. 65 (2008) 2507 – 2527
1420-682X/08/162507-21
DOI 10.1007/s00018-008-8082-6

Cellular and Molecular Life Sciences

Review

Mechanisms and structures of crotonase superfamily enzymes – How nature controls enolate and oxyanion reactivity

R. B. Hamed†, E. T. Batchelar†, I. J. Clifton* and C. J. Schofield*

Chemistry Research Laboratory, Department of Chemistry, University of Oxford, Mansfield Road, Oxford, OX1 3TA (UK), Fax: +44-1865-275-674, e-mail: ian.clifton@chem.ox.ac.uk, christopher.schofield@chem.ox.ac.uk

Received 18 January 2008; received after revision 25 March 2008; accepted 1 April 2008
Online First 13 May 2008

Abstract. Structural and mechanistic studies on the crotonase superfamily (CS) are reviewed with the aim of illustrating how a conserved structural platform can enable catalysis of a very wide range of reactions. Many CS reactions have precedent in the 'carbonyl' chemistry of organic synthesis; they include alkene hydration/isomerization, aryl-halide dehalogenation, (de)carboxylation, CoA ester and peptide hydrolysis, fragmentation of β-diketones and C-C bond formation, cleavage and oxidation. CS enzymes possess a canonical fold formed from repeated ββα units that assemble into two approximately perpendicular β-sheets surrounded by α-helices. CS enzymes often, although not exclusively, oligomerize as trimers or dimers of trimers. Two conserved backbone NH groups in CS active sites form an oxyanion 'hole' that can stabilize enolate/oxyanion intermediates. The range and efficiency of known CS-catalyzed reactions coupled to their common structural platforms suggest that CS variants may have widespread utility in biocatalysis.

Keywords. Crotonase superfamily, oxyanion hole, enolate intermediates, coenzyme A, β-oxidation, proteases.

Introduction

Enoyl-CoA hydratase, which was the first member of the crotonase superfamily (CS) to be identified, catalyzes the 'simple' addition of water to α,β-unsaturated coenzyme A (CoA) esters, an important step in fatty acid β-oxidation [1]. Subsequently, CS enzymes have been revealed to catalyze examples from all six classes of reaction defined in the Enzyme Commission (EC) classification scheme (Fig. 1, Table 1). The CS are defined by a common protein fold formed from repeated ββα units that assemble into two approximately perpendicular β-sheets surrounded by α-helices. Many CS catalyzed reactions have precedent in the 'carbonyl' chemistry of organic synthesis in which enol/enolate intermediates are a common feature. Many CS reactions feature the stabilization of an enolate/oxyanion intermediate *via* two backbone NH groups, analogous to the oxyanion hole (OAH) of proteases. CS reactions include alkene hydration/isomerization, aryl-halide dehalogenation, (de)carboxylation, CoA ester and peptide hydrolysis, fragmentation of β-diketones, C-C bond formation, cleavage and oxidation reactions (Fig. 2, Table 1).
Here we review structural and mechanistic studies on the CS, with the emphasis on those members for which crystal structures have been reported. We aim to illustrate how Nature has varied common structural features to evolve catalysts for a remarkably diverse

† These authors contributed equally to this work.
* Corresponding authors.

Table 1. Members of the crotonase superfamily (CS) discussed in this review, organized according to their EC classification numbers except for **XVII**–**XX**.

	Name (abbreviation)	EC no.	Biochemical role/*reaction class*	Oligomerization state/type of association fold	Proposed oxyanion hole forming residues	PDB code for apo protein
I	3,5-Dihydroxyphenylglyoxylate synthase (**DpgC**)	1.2.3.-	Cofactor independent dioxygenase involved in vancomycin biosynthesis/ *oxygenase*	Hexamer	Ile 235 and Gly 296	2np9 [5]
II	Transcarboxylase 12S (**TC 12S**)	2.1.3.1	Class III biotin dependent carboxylase/*carboxyltransferase*	Hexamer/inter-trimer	Ala 143 and H_2O bound to Ala 180 and Ala 183	1on3 [27]
III	*Anabaena* β–Diketone hydrolase (**ABDH**)	3.7.1.7	Secondary metabolism/*hydrolase*	Hexamer/self-association	Not assigned	2j5g [96]
IV	6-Oxocamphor hydrolase (**6-OCH**)	3.7.1-	Camphor metabolism/*hydrolase*	Hexamer/self-association	Not assigned	1o8u [94]
V	4-Chlorobenzoyl-CoA dehalogenase (**4-CBD**)	3.8.1.7	4-Chlorobenzoate degradation/ *dehalogenase*	Trimer/V-shaped intra-trimer	Phe 64 and Gly 114	1nzy [11]
VI	Methylmalonyl-CoA decarboxylase (**MMCD**)	4.1.1.41	*E. coli* succinate catabolism/ *decarboxylase*	Hexamer (dimer of trimers)/self-association	His 66 and Gly 110	1ef8 [17]
VII	Glutaconyl-CoA decarboxylase-α subunit (**Gcdα**)	4.1.1.70	Class II biotin dependent carboxyl-transferase/ *carboxyltransferase*	Dimer/inter-trimer	Val 151 and Gly 194	1pix [13]
VIII	ECH_2 Decarboxylase domain of CurF (**CurF**)	4.1.1.-	Curacin A biosynthesis/ *decarboxylase*	Trimer*	Ala 78 and Gly 118	2q2x [69]
IX	Naphthoate synthase (**MenB**)	4.1.3.36	Vitamin K2 biosynthesis/*synthase*	$(\alpha_3)_2$ Hexamer/ inter-trimer	Gly 125 and Gly 161	1q52 [20]
X	Adenine, Uracil binding ECH homologue (**AUH**)	4.2.1.17	RNA binding and hydration of 2-*trans*-enoyl-CoA/ *hydratase*	Hexamer (dimer of trimers)/ V-shaped intra-trimer	Ala 141 and Gly 186 (by analogy to ECH)	1hzd [61]
XI	Enoyl-CoA hydratase (**ECH**)	4.2.1.17	Fatty acid β-oxidation/*isomerase*	Hexamer (dimer of trimers)/ V-shaped intra-trimer	Ala 98 and Gly 141	1dub [32]
XII	Dienoyl-CoA isomerase (**DCI**)	4.2.1.17	Fatty acid β-oxidation/*isomerase*	Hexamer (dimer of trimers)/ V-shaped intra-trimer	Ile 117 and Gly 173	1dci [44]
XIII	Hydroxylcinnamoyl-CoA hydratase-lyase (**HCHL**)	4.2.1.101	Phenolic compound degradation/ *hydratase, lyase*	Hexamer (dimer of trimers)	Met 70 and Gly 120	2j5i [57]
XIV	Δ^3,Δ^2-Enoyl-CoA isomerase (**ECI**)	5.3.3.8	Auxiliary fatty acid β-oxidation pathway/*isomerase*	Hexamer/self-association	Ala 70 and Leu 126	1hno [40]
XV	Acetyl-CoA carboxylase carboxyltransferase subunit from yeast (**ACC CT**)	6.4.1.2	Regulation of fatty acid biosynthesis and metabolism/ *carboxyltransferase*	Dimer/inter-trimer	Not assigned	1od4 [15]
XVI	Carboxymethylproline synthase (**CarB**)	6.4.1.-	Carbapenem antibiotic biosynthesis/*decarboxylase, synthase, hydrolase*	Trimer of trimers/ self-association	Gly 62 and Met 108	2a7k [12]
XVII	The proteolytic subunit of caseinolytic protease (**ClpP**)	3.4.21.92	Serine protease/*hydrolase*	Tetradecamer	Gly 68 and Met 98	1tyf [108]
XVIII	Photosystem II D1 CTPase (**D1-CTPase**)	3.4.21.-	Serine protease/*hydrolase*	Monomer	Gly 318 and Ala 373	1fc7 [117]
XIX	Interphotoreceptor retinoid-binding protein (**IRBP**)	–	Protection of Vitamin A forms from oxidative and isomeric degradation/?	Monomer	Not assigned	1j7x [118]
XX	Tricorn protease (**Tricorn**)	3.4.21.-	Serine protease/*hydrolase*	Hexamer	Gly 918 and Asp 966	1k32 [121]

* The CurF ECH2 decarboxylase domain has been crystallized as a trimer but the oligomerization state of full length CurF has not been reported.

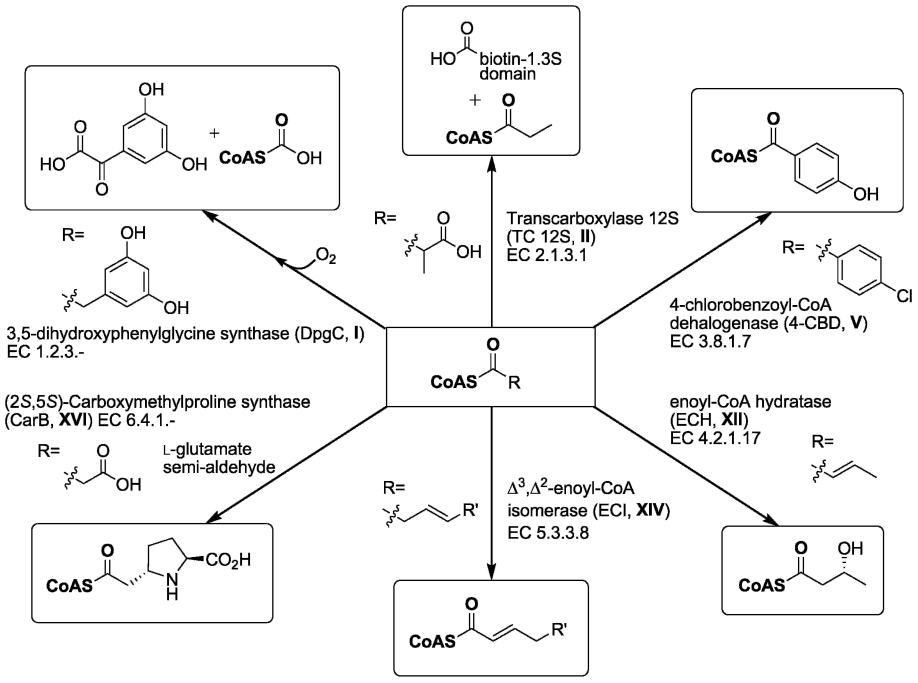


Figure 1. Reactions of the crotonase superfamily (CS) classified according to the Enzyme Commission (EC) nomenclature. Crotonases catalyze a diverse set of reactions with common CoA ester (in bold) substrates. 3,5-Dihydroxyphenylglyoxylate synthase (DpgC) (**I**), transcarboxylase 12S (TC 12S) (**II**) and carboxymethylproline synthase (CarB) (**XVI**) catalyze more than one different type of reaction, with only one type shown in this figure. Note: CoA-independent CS enzymes have also been identified.

set of reactions (for prior reviews see [2–4]). Throughout the review, in both text and figures, bold roman numerals correspond to the CS proteins as defined in Table 1. The discoveries that a cofactor-independent oxygenase (3,5-dihydroxyphenylglyoxylate synthase, DpgC, **I** [5] and a heterocycle forming enzyme (carboxymethylproline synthase, CarB, **XVI**) [6, 7] are members of the CS has recently extended the mechanistic range of the family. Bioinformatic analyses imply that CS enzymes are ubiquitous (Fig. 2) and it is reasonable to propose that their scope extends well beyond the reactions identified at present.

We hope that this review will help to stimulate further work on the CS, not only in terms of functional and structural/mechanistic studies, but also in the application of variants of the CS in biocatalysis.

Overview of CS structures

Overall architecture

While overall sequence similarities between individual CS proteins are often low, all members share a similar overall architecture. CS members possess a canonical crotonase fold, formed from repeated ββα units, that comprises two approximately perpendicular β-sheets surrounded by α-helices (Fig. 3A, B). In most cases the C-terminal α-helical domain enables oligomerization. The Structural Classification of Proteins Database [8] defines four structural families within the caseinolytic protease (Clp)/crotonase superfamily: (i) the Clp protease, Clp subunit family (**XVII**), (ii) the tail-specific protease, catalytic domain family (*e.g.*, **XVIII**–**XX**), (iii) the crotonase-like family (*e.g.*, **IV**–**VI**, **IX**-**XII**, **XIV**, **XVI**), and (iv) the biotin-dependent carboxylase carboxyltransferase domain family (*e.g.*, **II**, **VII**, **XV**).

Oligomerization

The oligomerization state of CS members varies (Table 1, Fig. 3C). In many CS enzymes, three monomeric subunits assemble into a characteristic homo-trimeric disk with tight interactions between monomers. These homo-trimeric disks usually dimerize, although the trimer–trimer interactions are not as tight as the intra-trimer contacts [9]. Crystallographic analyses have provided details of these interactions, however the apparent 'oligomerization' state observed in the crystallized state does not always reflect that in solution. Human mitochondrial enoyl-CoA isomerase (ECI, **XIV**) [10] exists in solution as a homo-trimer and also crystallizes as a homo-trimer. However, 4-chlorobenzoyl-CoA dehalogenase (4-CBD, **V**, Fig. 3C 3) and CarB (**XVI**, Fig. 3C 4) crystallize as a dimer of trimers (hexamer) and a trimer of trimers (nonamer), respectively, although both exist in solution, at least predominantly, as trimers [11, 12].

Not all CS enzymes possess the trimeric disk structure, for example some carboxyltransferase (CT) enzymes, which have two CS folds, are dimers [see *Carboxyltransferase subunit of biotin dependent carboxylases – ACC CT (**XV**), Gcdα (**VII**) and TC 12S (**II**)* below]. The CT glutaconyl-CoA decarboxylase α-subunit

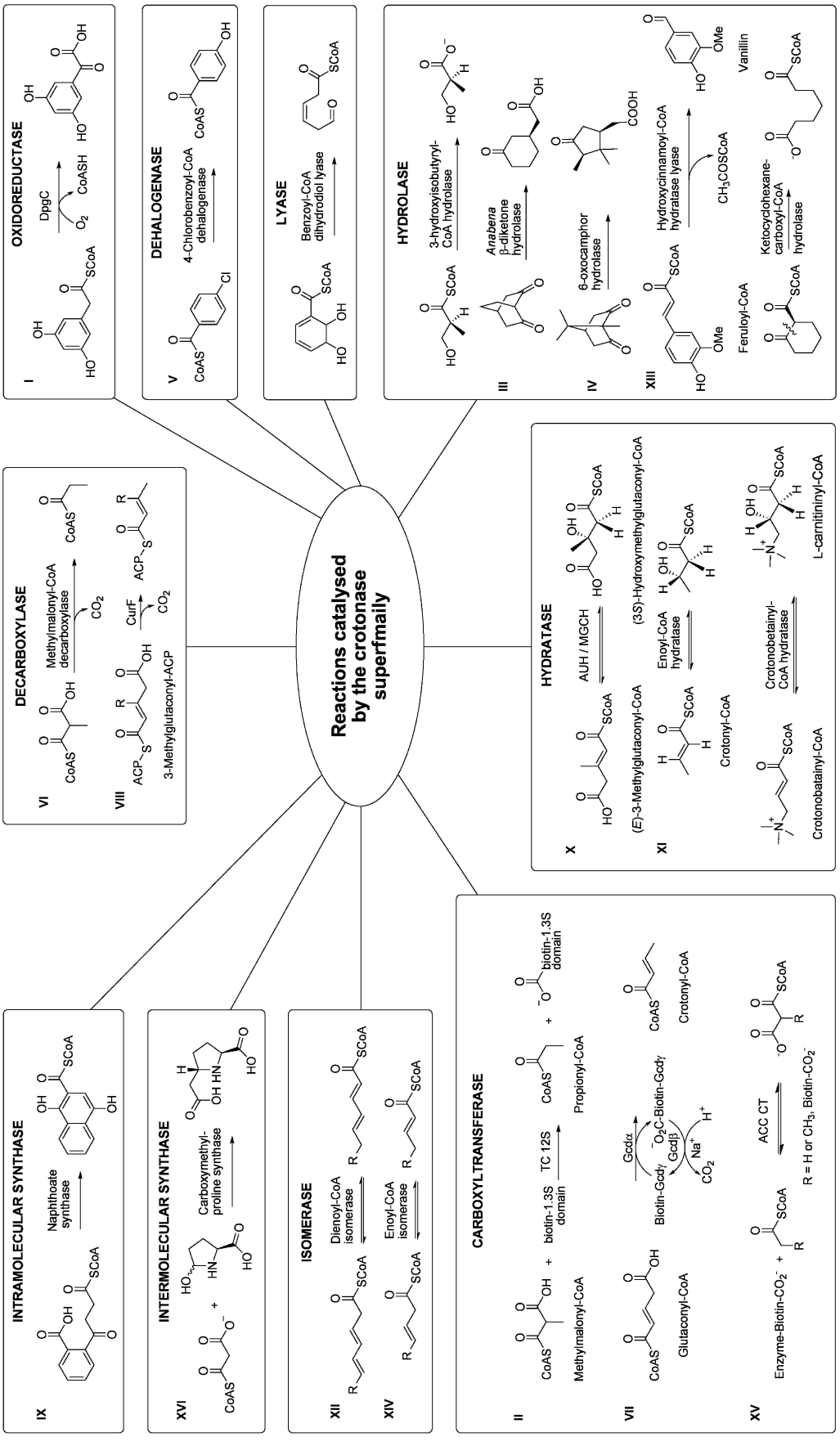


Figure 2. Summary of reactions catalyzed by CS members described in this review, classified according to the type of reaction catalyzed. Note the similarity between the chemistry of the CS and that of synthetic 'carbonyl' chemistry.

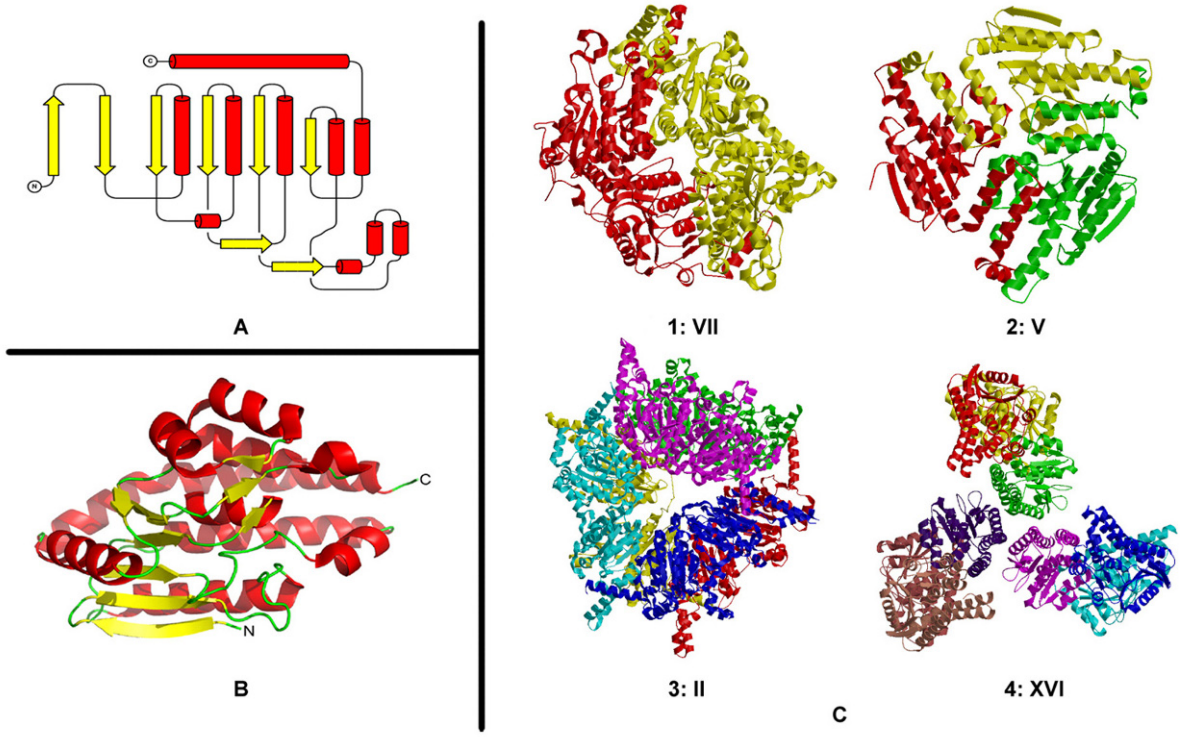


Figure 3. (*A*) Schematic topology of a CarB (**XVI**) monomer showing the repeated crotonase motif (ββα) of the CS fold. (*B*) A view of the CarB monomer (PDB code: 2a7k). (C) Oligomerization states of some crotonases; 1: glutaconyl-CoA decarboxylase α-subunit (Gcdα) (**VII**), dimer (PDB code: 1pix), 2: 4-chlorobenzoyl-CoA dehalogenase (4-CBD) (**V**), trimer (PDB code: 1nzy), 3: TC 12S, (**II**), hexamer [dimer of trimers] (PDB code: 1on3) and 4: CarB (**XVI**), trimer of trimers (PDB code: 2a7k). Figures were generated using TopDraw [129], Pymol [130], Molscript [131] and Raster3D [132].

(Gcdα, **VII**) from *Acidaminococcus fermentans* [13] is also dimeric (Fig. 3C 1), the CTs of acetyl-CoA carboxylase from *Staphylococcus aureus* and *Escherichia coli* [14] are tetrameric (dimer of dimers) and the CT domain of acetyl-CoA carboxylase (ACC) from *Saccharomyces cerevisiae* (ACC CT, **XV**) [15] is dimeric. Dimerization of the CT enzymes is important for stabilization and occurs in a head to tail manner [15, 16] (Fig. 3C).

It has been proposed that the canonical fold of many CS members is fundamentally identical except for inter-domain swapping of the C-terminal α-helical residues [17]. Domain swapping is proposed as a mechanism by which CS monomers may have evolved to form intertwined oligomers [18].

Members of the CS have been classified into three groups according to the relationship of the C-terminal α-helical domain of individual subunits with respect to the rest of the structure into those that undergo: (i) monomer self-association, (ii) intra-trimer associations or (iii) inter-trimer associations [19, 20]. In the monomer self-association fold, the C-terminal domain folds back over the core to cover the active site of its own monomer, for example as in *Anabaena* β-diketone hydrolase (ABDH, **III**), 6-oxocamphor hydrolase (6-OCH, **IV**), methylmalonyl-CoA decarboxylase (MMCD, **VI**), ECI (**XIV**), and CarB (**XVI**) oligomerization. In the case of the V-shaped intra-trimer association fold, the C-terminal domain protrudes away from the monomer to which it belongs to cover the active site of a neighboring monomer within the same trimer, for example, as in 4-CBD (**V**), enoyl-CoA hydratase (ECH, **XI**), adenine, uracil binding ECH homologue (AUH, **X**) and dienoyl-CoA isomerase (DCI, **XII**) oligomerization. In the inter-trimer association fold, the C-terminal domain crosses the trimer-trimer interface and forms part of the active site in the monomer of an opposing trimer, for example, as in naphthoate synthase (MenB, **IX**), transcarboxylase 12S (TC 12S, **II**, Fig. 3C 2), Gcdα (**VII**) and yeast acetyl-CoA carboxylase carboxyltransferase subunit (ACC CT, **XV**) oligomerization. Domain swapping classifications for DpgC (**I**), the N-terminal ECH_2 domain of CurF (CurF, **VIII**) and hydroxycinnamoyl-CoA hydratase-lyase (HCHL, **XIII**) have not been reported.

The oligomerization state of some CS members plays a clear role in substrate binding and/or catalysis: in the case of the biotin-dependent carboxylases (BDCs, **II**, **VII**, **XV**), the structures of the CoA complexes of these CT enzymes reveal their active sites are at the dimer interface [15, 16]. Specific oligomerization states do not appear to correlate to particular types of reaction catalyzed by the CS. However, the different possible assembly modes for oligomers may have helped to enable the evolution of the wide range of substrate and product selectivities in the CS, possibly by domain swapping.

Active sites

The CS is one of a growing number of enzyme superfamilies that demonstrate how a common polypeptide fold can be used as a scaffold to catalyze a wide variety of reactions (for reviews see [2, 3]). A common feature of most CS mechanisms (outlined in Fig. 4) is an intermediate with a negatively charged oxygen atom (oxyanion). These intermediates include enolates and 'tetrahedral' intermediates involved in hydrolysis and other reactions. Stabilization of oxyanion intermediates in enzyme catalysis was first studied in detail with the serine (and cysteine) proteases [21, 22]. The enolate and oxyanion tetrahedral intermediates involved in the formation and breakdown of acyl enzyme (ester) complexes in catalysis by CS enzymes are stabilized in an OAH formed in most, but not all, cases by two backbone amide NH groups [11, 23] (Table 1, Figs. 5A and 6). In some cases there is uncertainty as to which residues form the OAH, in part due to the diversity of reactions catalyzed by the CS, and in part due to the limited number of substrate structures that are presently available.

Some enzymes possessing classic serine protease folds and catalytic triads are able to catalyze non-protease reactions (see *e.g.*, [24]). However, the OAH containing CS active sites likely support an even wider range of reaction types than the serine proteases; indeed the CS even contains some proteases (*e.g.*, the Clp proteases, Fig. 2) [3, 4].

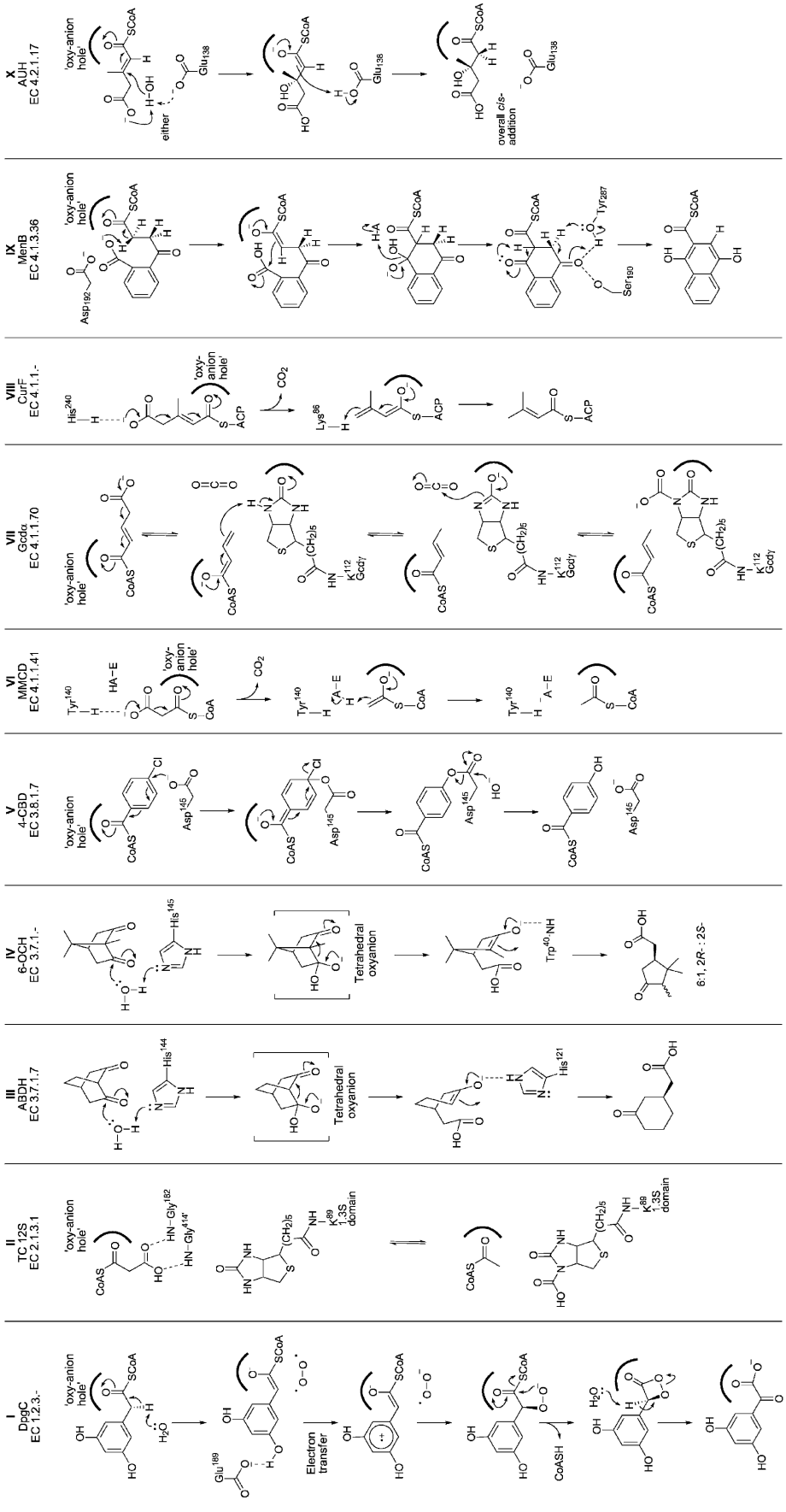
A common mechanistic feature for all CS members employing CoA thioester substrates is polarization of the thioester carbonyl in the OAH. This polarization enables formation of thioester enolates and aids in decarboxylation reactions. The thioester enolates can be formed by decarboxylation, deprotonation, fragmentation or addition (Figs. 2 and 4). The OAH is also important in stabilizing oxyanion intermediates in the CS members that do not employ CoA thioesters. Apart from the OAH, no polar functional groups are highly conserved in the CS active site. However, general acid/base catalysis is commonly proposed to be mediated by aspartyl or glutamyl residues (*e.g.*, **I**, **V**, **IX**, **X**, **XI**, **XII**, **XIV**, **XVI**) or histidyl or lysyl residues (*e.g.*, **III**, **IV**, **VI**, **VIII**) (Fig. 4). The CS may thus be an example of how a common structural fold with limited conserved polar active site features (*e.g.*, an OAH) can evolve into enzymes that catalyze very different types of reaction with little apparent primary sequence similarity (often less than 20%) [25]. In support of the idea of a common platform on which activities can be relatively easily evolved or interchanged, there is experimental evidence that some of the reaction types catalyzed by CS members can be interchanged, *e.g.*, mutation of 4-CBD (**V**) to introduce active site residues present in ECH (**XI**) results in a 4-CBD (**V**) mutant with previously absent hydratase activity [26] (see *CoA-dependent CS members and their mechanisms* below).

Structures of all CS members crystallized in the presence of a CoA substrate (or analogue) show the active sites to be spatially similar with respect to binding of the CoA portion of the substrate [19]. The CoA portion of CS substrates is usually observed to be bound in the extended binding pocket in a U-shape, centered around the pyrophosphate group (Fig. 5A). The substrates are bound at the interface between monomers [2], except for the structures reported for MMCD (**VI**) and human mitochondrial ECI (**XIV**) (both of which have the C-terminal self-association fold and their substrate-binding sites are completely defined within a single monomer) [10, 17]. Several CS structures [*e.g.*, 4-CBD (**V**), ECH (**XI**) and DCI (**XII**)] bind substrates at the interface between monomers within the same trimer, while TC 12S (**II**) binds its substrate at the interface between monomers in opposing trimers. Most CS hexamers (dimer of trimers) are formed such that the six active sites are at the outer faces of the trimers, *i.e.*, directed away from the trimer–trimer interface. However, the TC 12S hexamer (**II**) is unusual because its six active sites are located at the trimer–trimer interface [27].

CoA-dependent CS members and their mechanisms – ECH (XI), ECI (XIV), DCI (XII), 4-CBD (V), DpgC (I), MenB (IX), HCHL (XIII) and AUH (X)

Rat enoyl-CoA hydratase (ECH, XI)

Three crotonases are involved in the ubiquitous fatty acid β-oxidation pathway: ECH (**XI**), ECI (**XIV**) and DCI (**XII**) (Fig. 7). ECH (**XI**) catalyzes the reversible *syn*-addition of a water molecule across the double bond of a *trans*-2-enoyl-CoA (*e.g.*, crotonyl-CoA) thioester to give a β-hydroxyacyl-CoA thioester [1, 28] (Fig. 7). ECH (**XI**) catalysis is extremely efficient:


I DpgC EC 1.2.3.-
II TC:12S EC 2.1.3.1
III ABDH EC 3.7.1.7
IV 6-OCH EC 3.7.1.-
V 4-CBD EC 3.8.1.7
VI MMCD EC 4.1.1.41
VII Gcdα EC 4.1.1.70
VIII CurF EC 4.1.1.-
IX MenB EC 4.1.3.36
X AUH EC 4.2.1.17
'oxy-anion hole'
Tetrahedral oxyanion
6:1, 2R-:2S-
overall cis-addition
either
Electron transfer
CoASH

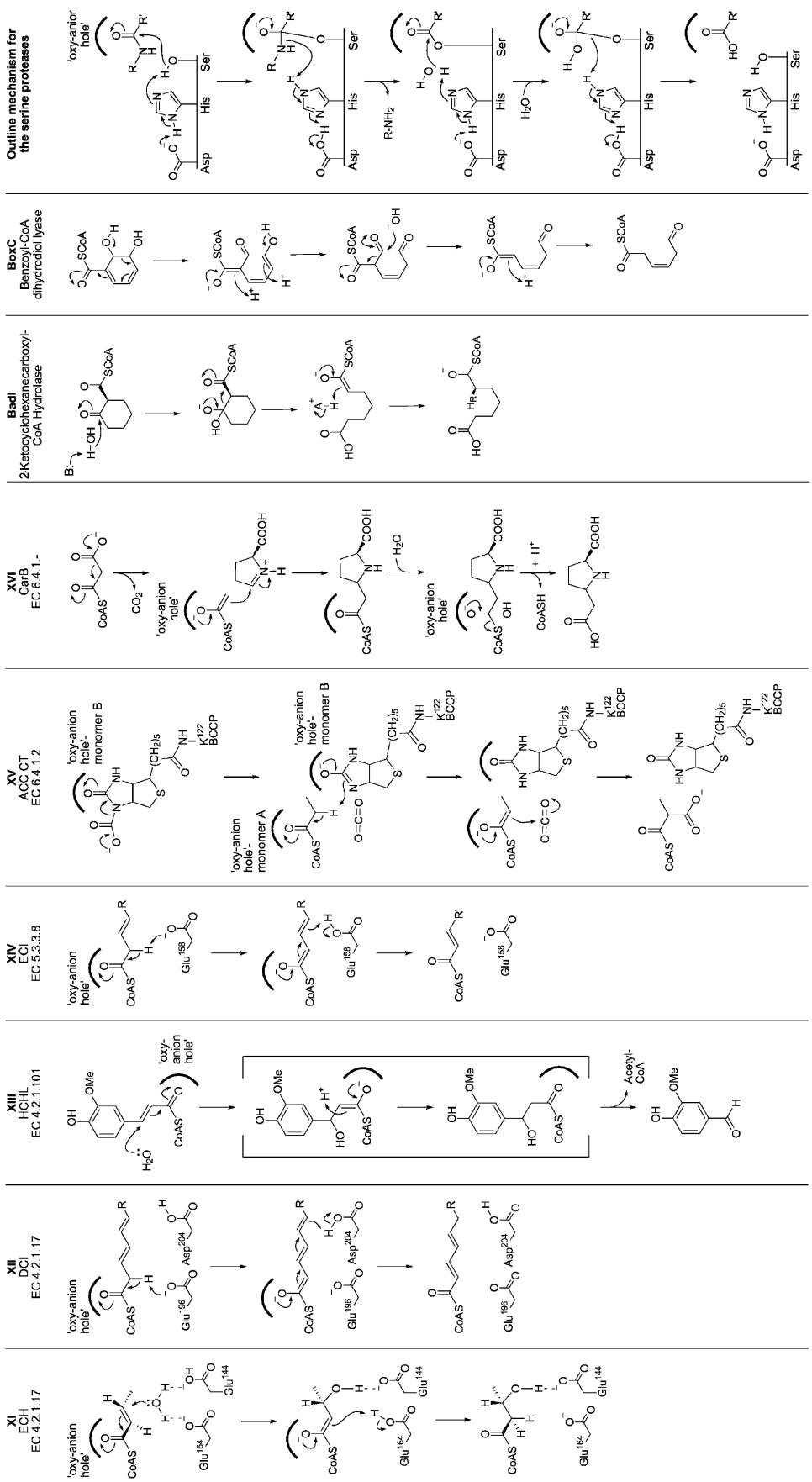


Figure 4. Outline reaction mechanisms for CS enzymes discussed in this review. These mechanisms have been proposed on the basis of reported solution and crystallographic studies (see text for discussion and references). Key residues and their roles are highlighted. The common oxyanion hole (OAH) is highlighted – for details of which residues form the OAH see Table 1. Also shown for comparison is an outline mechanism for the serine proteases. Note the importance of the OAH/intermediates. A related mechanism is proposed to apply to the caseinolytic (Clp) CS proteases.

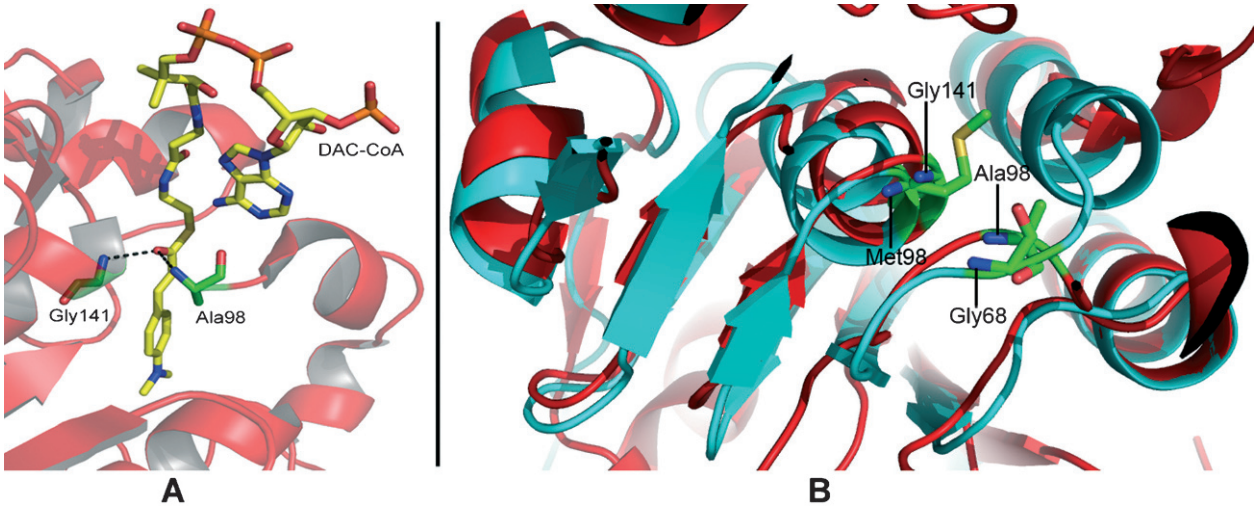


Figure 5. (*A*) View of enoyl-CoA hydratase (ECH, **XI**) complexed with the substrate 4-dimethylaminocinnamoyl-CoA (DAC-CoA) (PDB code: 1ey3) showing the OAH forming residues Ala98 and Gly141. Distances from the backbone NH to the substrate thioester carbonyl are 2.88 Å (Ala98) on right and 2.89 Å (Gly141) on left. Note the characteristic 'U-shaped' binding of the CoA adenosine diphosphate group. (*B*) Superimposed monomers of ECH (red) and the proteolytic subunit of Clp (ClpP, turquoise) showing the conserved location of OAH-forming residues: Ala 98 and Gly 141 for ECH (**XI**, PDB code 1dub) and Gly68 and Met98 for ClpP (**XVII**, PDB code 1tyf). Note the structural homology between the two monomers. Some residues have been removed for clarity. The figure was generated using Pymol [130] and DaliLite [107].

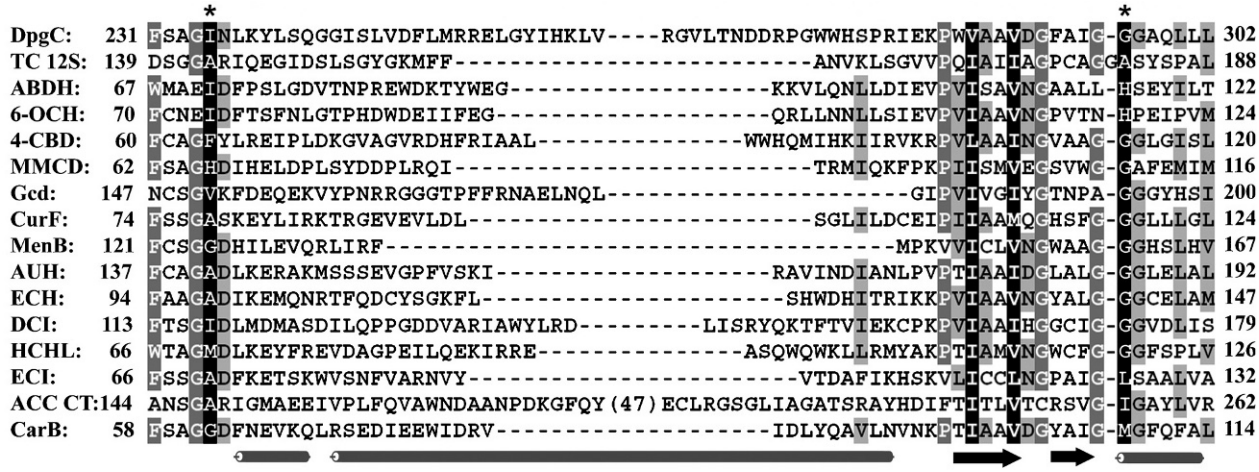


Figure 6. Structure based sequence alignment of selected CS members (in the same order as that in Table 1). The OAH forming residues are denoted by an asterisk. The alignment was prepared using the protein structure comparison service SSM [133] and the Genedoc programme [134]. α-Helices (cylinders) and β-strands (arrows) at the bottom of the figure represent the assigned secondary structure of CarB.

when crotonyl-CoA is used as substrate, the reaction rate is near the diffusion limit [29]. The stereoselectivity of ECH (**XI**) catalysis varies: animal mitochondrial ECH (**XI**) forms (*S*)-3-hydroxyacyl-CoA [30], whereas an ECH from *Aeromonas caviae* forms (*R*)-3-hydroxyacyl-CoA [31]. ECH (**XI**) was the first CS member for which a crystal structure was solved by Engel and co-workers in 1996 [32]; the structure rationalized previous kinetic and mutagenesis studies. Two glutamyl residues (Glu144 and Glu164) are essential for ECH (**XI**) catalysis [33, 34]. One proposed mechanism for ECH (**XI**) involves conjugate addition of water with general acid/base catalysis from Glu144 and Glu164 with polarization of the thioester carbonyl group by the OAH (Fig. 4). Kinetic studies coupled to crystallographic analyses support a mechanism employing concerted *syn* addition of water [35, 36].

ECH (**XI**) also has enoyl-CoA isomerase (ECI, **XIV**) activity at approximately 1/5000 the level of its hydratase activity. Independent mutation of Glu144 and Glu164 to alanyl residues decreased the level of isomerase activity 10- and 1000-fold, respectively [37], revealing that these residues are important in both the

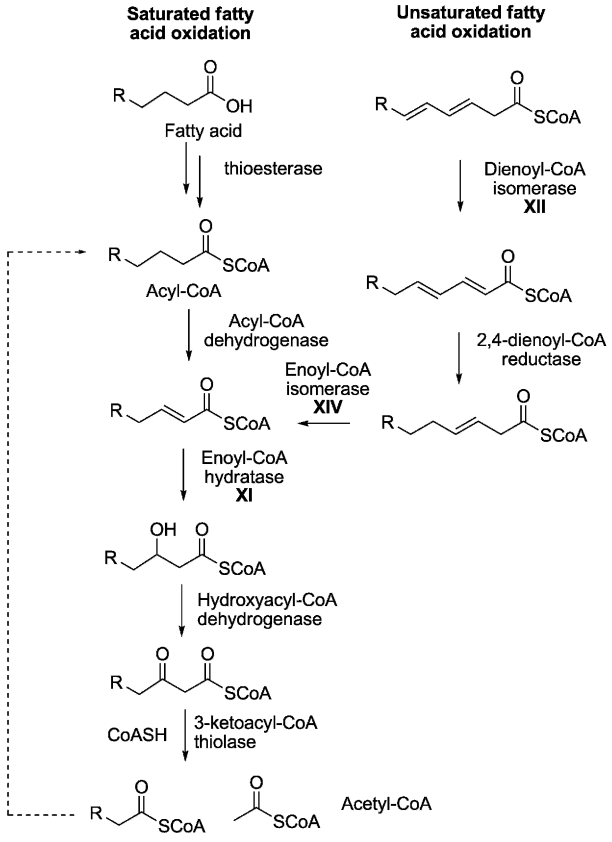


Figure 7. The fatty acid β-oxidation pathway showing the roles of DCI (**XII**), ECI (**XIV**) and ECH (**XI**). Adapted from Fig. 1 of Modis et al. [44].

hydratase and isomerase activities. For a review on ECH, see Agnihotri and Liu [38].

Δ^3,Δ^2-Enoyl-CoA isomerase (ECI, XIV)

ECI (**XIV**) catalyzes isomerization of 3-*cis*- or 3-*trans*-enoyl-CoA to 2-*trans*-enoyl-CoA, which, like enoyl-CoA hydratase (ECH, **XI**), is an essential part of the fatty acid β-oxidation pathway [39] (Fig. 7). ECIs (**XIV**) can be grouped into three classes: monofunctional mitochondrial, monofunctional peroxisomal and multifunctional enzymes [40]. ECIs (**XIV**) accept a range of acyl-chain length substrates but all need a C-3,C-4 unsaturated CoA thioester substrate [41, 42]. The ECI (**XIV**) reaction involves the abstraction of a proton at C-2, electron redistribution and then protonation at C-4. This reaction is thermodynamically favorable as it converts two isolated double bonds into an extended α,β-unsaturated-conjugated system. The OAH probably serves to increase the acidity of the C-2 proton to be abstracted and stabilize the enolate intermediate (Fig. 4). Unlike ECH (**XI**) [32], ECI (**XIV**) only contains a single glutamyl residue (Glu158) in its active site, which is proposed to both deprotonate at C-2, and after electronic rearrangement, protonate at C-4. The substrate is bound in a large hydrophobic pocket around a buried tryptophan residue (Trp38) thought to be involved in binding the acyl side chain of the substrate [40].

Dienoyl-CoA isomerase (DCI, XII)

DCI (**XII**) catalyzes the isomerization of 3,5-dienoyl-CoA into 2,4-dienoyl-CoA [43]. The ability of DCI (**XII**) to isomerize unsaturated fatty acids decreases with increasing length of the fatty acid side chain [44]. Analogous to the ECI (**XIV**) mechanism, that proposed for DCI (**XII**) (Fig. 4) involves initial proton abstraction from C-2, to form an OAH-stabilized CoA thioester enolate, followed by tautomerization through the conjugated system and protonation at C-6. A crystal structure of DCI (**XII**) revealed similarities with the ECI (**XIV**) active site, including the OAH [44].

Because of the extended conjugated system in the unsaturated side chain of the substrate, in DCI (**XII**) catalysis the enolate intermediate can potentially react with a choice of protonation sites (on C-2, C-4 or C-6). Only three hydrophilic residues are present in the hydrophobic active site pocket: Asp176, Glu196 and Asp204. Glu196 is proposed to be the C-2 proton abstractor and Asp204 the C-6 proton donor. The need for separate abstractors and donors in DCI (**XII**), compared to ECI (**XIV**) where there is only one (Glu158), presumably reflects the increased distance between the abstraction and donation sites. Glu196 is also hydrogen bonded to Asp176, and this interaction may help Glu196 to achieve the appropriate pKa for catalysis. Glu196 of DCI (**XII**) is similarly positioned to the catalytically important Glu164 of ECH (**XI**) [33, 34].

4-Chlorobenzoyl-CoA dehalogenase (4-CBD, V)

4-CBD (**V**) catalyzes the nucleophilic aromatic substitution of 4-chlorobenzoyl-CoA to give 4-hydroxybenzoyl-CoA [45]. This reaction is part of the 4-chlorobenzoate degradation pathway in *Pseudomonas* species CBS3 that is proposed to have evolved in response to the release of synthetic chlorinated organic compounds into the environment [11]. The 4-CBD (**V**) reaction (Fig. 2) is an example of an enzymatically catalyzed nucleophilic aromatic substitution (S_NAr) reaction that occurs in synthetic chemistry *via* an addition-elimination mechanism (Fig. 4). The formation of the tetrahedral addition intermediate and activation of the H_2O molecule that effects the hydrolysis appears to have been decoupled into two separate steps by 4-CBD (**V**) [46, 47]. Initial attack onto the 4-chlorobenzoyl-CoA substrate is by a suitably positioned aspartyl residue (Asp145), which

reacts to form a covalently linked ester intermediate [11, 48]. Asp145 of 4-CBD (**V**) is analogous to Asp204 of DCI (**XII**) but these residues play different roles in catalysis: Asp145 is a nucleophile and Asp204 is a general acid/base. The OAH of 4-CBD (**V**) likely assists in activating the substrate to attack by Asp145 and stabilizing the resultant thioester enolate intermediate [11]. The intermediate can then collapse by eliminating a chloride ion to give an aromatic ester intermediate. Hydrolysis of the Asp145-4-hydroxybenzoyl-CoA ester intermediate is likely catalyzed by the only charged residue in the vicinity, His90, activating a water molecule [11]. This final hydrolysis of the ester intermediate is rate limiting [46]. The conjugated portion of the intermediates is proposed to be stabilized by stacking interactions involving Phe64, Trp89 and Trp137 [11].

3,5-Dihydroxyphenylglyoxylate synthase (DpgC, I)

DpgC (**I**) catalyzes the oxygen-dependent conversion of 3,5-dihydroxyphenylacetyl-CoA (DPA-CoA) to 3,5-dihydroxyphenylglyoxylate (DPGx) [49, 50] and is the first identified example of oxidation/reduction chemistry catalyzed by the CS. In *Actinomycetes*, DPGx is the penultimate intermediate in the biosynthesis of 3,5-dihydroxyphenylglycine (DPG), an amino acid in the nonribosomal production of the vancomycin/teicoplanin family of glycopeptide antibiotics [49]. The C-terminal domain of DpgC (**I**) possesses the CS fold [5]. DpgC (**I**) belongs to a small class of dioxygenases that are not dependent on an accessory cofactor or metal ion [49]. In synthetic chemistry, conditions for the reaction of enolates with dioxygen have been reported but such reactions are not routinely used. The discovery of oxygenases with neither a metal nor a cofactor raises the question of how these enzymes activate oxygen. The crystal structure of DpgC (**I**) in complex with a substrate analogue suggests that it is the enzyme-bound substrate itself that activates the oxygen molecule [5]. The DpgC (**I**) reaction involves both the four-electron oxidation of a benzylic carbon in DPA-CoA and the cleavage of the thioester bond to generate free CoA [50, 51]. The DpgC mechanism is proposed [5, 52] to involve stereospecific α-deprotonation of substrate, facilitated by an ordered water molecule, giving an OAH-stabilized intermediate. Molecular oxygen bound in a hydrophobic pocket and oriented by a hydrogen bond from the backbone NH of Ile324 is positioned to react with the enolate. Transfer of an electron from the enolate to the triplet state oxygen molecule is proposed to form a radical pair which can then react in a spin-allowed C-O bond-forming process to give a peroxide intermediate. The peroxide intermediate then displaces the CoA thiolate *via* an addition-elimination mechanism involving the OAH, to give a 1,2-dioxetanone. The α-hydrogen of the 1,2-dioxetanone undergoes deprotonation analogous to that of the pro-(*R*)-hydrogen in substrate DPA-CoA, and generates the DPGx product. The radical cation intermediate is proposed to be stabilized by partial deprotonation of the phenolic 3-OH group by Glu189.

1,4-Dihydroxy-2-naphthoyl-CoA synthase (MenB, IX)

MenB (**IX**) catalyzes the production of 1,4-dihydroxy-2-naphthoyl-CoA from *O*-succinylbenzoyl-CoA [53–55], an example of an enzyme catalyzed Dieckmann (or intramolecular Claisen) reaction (Fig. 2). MenB (**IX**) is involved in the biosynthesis of menaquinone (vitamin K_2), the sole quinone in *Mycobacterium tuberculosis*, a compound proposed to be essential for the survival of these bacteria. In humans, vitamin K is a blood-clotting agent but must either be ingested *via* the diet or produced by intestinal bacteria. Menaquinone biosynthesis is therefore a potential target for drugs to treat tuberculosis [20]. Crystallographic analyses reveal that MenB (**IX**) is unusual as it is the only CS non-carboxyltransferase whose C-terminal domain crosses the trimer-trimer interface [19, 20] (see *Biotin-independent CS decarboxylases* below for CS carboxyltransferases). To date, the MenB (**IX**) reaction is a rare example of intramolecular C-C bond formation within the CS (along with CarB, **XVI**), although other members catalyze C-C fragmentation. The MenB (**IX**) reaction is initiated by deprotonation α to the thioester carbonyl, proposed to be intramolecular *via* the substrate C-2 carboxylate with assistance from the side chain carboxyl of Asp192 [20]. The resultant thioester enolate, stabilized by the OAH, can then react with a C-2 carboxylic acid to form a bicyclic structure *via* an addition-elimination mechanism, in which Asp192 may act as a general acid/base catalyst. Tautomerization of both ketones to form the extended unsaturated naphthoate core is thermodynamically favorable. Stereospecific retention of the pro-(3*R*)-proton suggests that keto-enol tautomerization occurs on the enzyme, possibly involving the participation of Tyr287 [20].

Hydroxycinnamoyl-CoA hydratase-lyase (HCHL, XIII)

HCHL (**XIII**) catalyzes consecutive reactions that convert the acyl-CoA thioester of ferulic acid [3-(4-hydroxy-3-methoxy-phenyl)-prop-2-enoic acid] into vanillin (4-hydroxy-3-methoxy-benzaldehyde) [56] (Fig. 2). Vanillin is a commercially important aldehyde desired for its flavor and aroma, and is an intermediate in the degradation of ferulic acid, a

phenolic compound. The degradation of phenolic compounds is environmentally and economically important in the decay of wood and the disposal and recycling of plant wastes [57]. The HCHL (**XIII**) catalyzed reaction occurs in two steps: the first is a conjugate addition of water to an α,β-unsaturated CoA thioester similar to the enoyl-CoA hydratase (ECH, **XI**) reaction; the second is a retro-aldol elimination of acetyl-CoA to give vanillin (Fig. 4). A crystal structure of HCHL (**XIII**) reveals structural similarity to ECH (**XI**) [57]. In addition to an OAH, the structure also revealed a water molecule at ~3.5 Å from the benzylic C-atom hydrogen bonded to the carboxyl OH of Glu143 and the backbone NH of Gly151 [57], suggesting these residues may be responsible for activating a water molecule for the conjugate addition. The proton source for keto-enol tautomerization is unknown but protonation and tautomerization must precede the retro-aldol reaction which leads to C-C fragmentation. Only Ser123 and Glu143 of the active site residues are suitably positioned and of the appropriate reactivity to assist in the retro-aldol reaction [57].

RNA-binding protein adenine, uracil binding ECH homologue (AUH, X)/3-methylglutaconyl-CoA hydratase (MGCH)

3-Methylglutaconyl-CoA hydratase (MGCH) catalyzes the *syn*-hydration of (*E*)-3-methylglutaconyl-CoA to (3*S*)-hydroxymethylglutaryl-CoA, the fifth step in leucine catabolism. MGCHs have been isolated from *Homo sapiens* [58], *Pseudomonas putida* [59] and from *Acinetobacter* sp. [60]. The human MGCH is also known as AUH protein. AUH (**X**) is a bifunctional protein that has two distinct activities: binding to AU-rich elements of mRNA and ECH-like activity [58, 61]. AUH deficiency in humans results in the metabolic disease type I methylglutaconic aciduria (MGA1), characterized by the excessive urinary excretion of 3-methylglutaconic acid. MGA1 patients have mutations within the human AUH-encoding gene. These mutations result in reduced or no MGCH activity [62, 63]. A fungal metabolic model of MGA1 demonstrated that leucine metabolites accumulating in AUH-deficient strains are toxic [64]. Bacterial MGCHs catalyze hydration of enoyl-CoAs despite having only one glutamyl residue in their active sites (Glu138 in case of MGCH from *Pseudomonas putida*). This contrasts with the model for enoyl-CoA hydratase (ECH, **XI**) catalysis as well as mammalian MGCHs which, like ECH (**XI**), have two active site glutamyl residues homologous to Glu144 and Glu164 in ECH (**XI**). 3-Methylglutaconyl-CoA substrate analogues without a γ-carboxylate act as competitive inhibitors of bacterial MGCH from *Pseudomonas putida* [59]. It is proposed that with bacterial MGCH, the substrate carboxylate group effectively replaces one of the active site glutamyl residues in the reactions catalyzed by ECHs (**XI**) and the eukaryotic MGCHs [59].

Biotin-independent CS decarboxylases – CarB (XVI), MMCD (VI) and CurF (VIII)

(2*S*,5*S*)-Carboxymethylproline synthase (CarB, XVI)

CarB (**XVI**) catalyzes the production of (2*S*,5*S*)-carboxymethylproline from (*S*)-glutamate semi-aldehyde and malonyl-CoA [6]. (2*S*,5*S*)-Carboxymethylproline is an early stage intermediate in the biosynthesis of carbapenem-3-carboxylate, the simplest of the medicinally important carbapenem antibiotic family. CarB (**XVI**) catalysis is unique to date within the CS as it catalyzes three different types of reaction within the same monomeric active site: malonyl/methylmalonyl-CoA decarboxylation (EC 4.1.1.-), carbon-carbon bond formation (EC 6.4.1.-) and thioester hydrolysis (EC 3.1.2-). The mechanism of these steps is proposed to proceed *via* decarboxylation of malonyl-CoA to generate the canonical acyl-CoA enolate, which reacts either with the ring-opened or closed form of (*S*)-glutamate semi-aldehyde to give the CoA ester of (2*S*,5*S*)-carboxymethylproline, which undergoes subsequent hydrolysis. Condensation onto the closed ring is proposed to be more likely at basic pH [7, 65]. If condensation onto the open chain glutamate semi-aldehyde occurs, CarB (**XVI**) will also catalyze a conjugate-addition type C-N bond forming reaction (EC 6.3.4.-) [6, 7]. CarB (**XVI**) contains the conserved OAH residues, which are proposed to be involved in decarboxylation, ring closure [if the reaction proceeds *via* the open form of (*S*)-glutamate semi-aldehyde], and thioester hydrolysis. The hydrolysis mechanism is unlikely to proceed *via* an oxygen-linked anhydride as proposed for 4-chlorobenzoate dehalogenase (**V**) [12] and 3-hydroxyisobutyryl-CoA hydrolase [66]. A homologue of CarB (**XVI**), ThnE, is present in the biosynthetic gene cluster for the C-2,C-6 functionalized carbapenem thienamycin [67], suggesting that thienamycin biosynthesis proceeds *via* a pathway related to that of carbapenem-3-carboxylate.

Methylmalonyl-CoA decarboxylase (MMCD, VI)

MMCD (**VI**) catalyzes the decarboxylation of (*S*)-methylmalonyl-CoA (MMCoA) to yield propionyl-CoA [68] and provides a pathway for the decarboxylation of succinate in *E. coli* [17]. Tyr140 of MMCD is proposed to bind the carboxylate group of MMCoA and assist in decarboxylation. An MMCD crystal

structure reveals an OAH but no clear residue serving as a proton donor to the α-carbon to generate propionyl-CoA was identified [17].

ECH_2 decarboxylase domain of CurF (CurF, VIII)

The N-terminal ECH_2 domain of the multifunctional protein CurF (CurF, **VIII**) from *Lyngbya majuscula* catalyzes the decarboxylation of the 3-methylglutaconyl-acyl carrier protein (ACP) complex to give 3-methylcrotonyl-ACP, a precursor of the cyclopropane ring of curacin A [69]. Curacin A is a mixed polyketide/non-ribosomal peptide having potent antimitotic and antiproliferative activities [70]. CurF (**VIII**) is the first CS member shown to act on ACP-linked substrates. Putative ECH_2-like decarboxylases are encoded within the 3-hydroxy-3-methylglutaryl-CoA synthase cassettes present in gene clusters encoding for the biosynthestic enzymes/proteins of a variety of polyketides, *e.g.*, bacillaene (PksI) [71, 72], virginiamycin M (VirE) [73], myxovirescin A (TaY) [74], mupirocin (MupK) [75], jamaicamide (JamJ) [76] and pederin (PedI) [77].

The hydrophobic active site of CurF includes only three polar side chains. Two of these, Lys86 and His240, are crucial for catalytic activity, but Tyr82 was shown to be nonessential by site-directed mutagenesis [69]. His240 is proposed to orientate the substrate carboxyl properly for fragmentation [69]. In CarB (**XVI**), His229 is similarly positioned to His240 of CurF (**VIII**), and is also proposed to bind to its substrate carboxylate [12]. An analogous histidyl residue does not exist in MMCD [17].

Carboxyltransferase subunit of biotin-dependent carboxylases – ACC CT (XV), Gcdα (VII) and TC 12S (II)

The carboxyltransferase (CT) subunit of biotin-dependent carboxylases (BDCs) contains two CS folds [78] (Fig. 8 **II**, **VII** and **XV**). BDCs comprise three components: a biotin carboxylase (BC), a carboxyltransferase (CT) and a biotin carboxyl carrier protein (BCCP), which contains the biotin molecule covalently attached to the ε-N of a lysyl residue [79]. Class 1 BDCs require ATP, Mg^{2+} and HCO_3^- (*e.g.*, ACC CT, **XV**) to catalyze carboxylation of acyl enolates; class 2 BDCs couple substrate decarboxylation with sodium transport in anaerobes (*e.g.*, Gcdα, **VII**); and the class 3 BDC (transcarboxylase from *Propionibacterium shermanii*), is a multienzyme complex that couples two carboxylation reactions, transferring a carboxylate from MMCoA to pyruvate, yielding propionyl-CoA and oxaloacetate [27, 80–82].

The carboxyltransferase domain of acyl-CoA carboxylases (ACC CT, XV)

Acetyl-CoA carboxylase (ACC) and propionyl-CoA carboxylase (PCC) catalyze the carboxylation of acetyl-CoA and propionyl-CoA to generate malonyl-CoA and methylmalonyl-CoA (MMCoA) respectively. ACC and PCC are examples of class 1 BDCs. Crystal structures of A/PCC CT subunits have been determined from bacteria and eukaryotes: the hexameric CT of propionyl-CoA carboxylase from *Streptomyces coelicolor* [16], the hexameric CT of acyl-CoA carboxylase from *Mycobacterium tuberculosis* (MtAccD5) [83], the dimeric CT of ACC from *Saccharomyces cerevisiae* (yeast-CT, **XV**) [15] and the tetrameric (dimer of dimers) CTs from *Staphylococcus aureus* and *E. coli* [14]. Eukaryotic ACCs are multifunctional enzymes which contain all three BDC components in one polypeptide [84]. In contrast, three different subunits (BC, BCCP, and CT) make up the *E. coli* ACC [84]. In actinomycetes and *Mycobacterium tuberculosis*, ACC consists of two polypeptides: an α-subunit containing the BC and BCCP domains and a β-subunit corresponding to the CT domain [85]. The ACC reaction involves two stages: firstly, BC catalyzes the transfer of the carboxyl group to biotin forming carboxybiotin. Secondly, the CT catalyzes the transcarboxylation between carboxybiotin and acetyl/propionyl-CoA to form malonyl/methylmalonyl-CoA. The carboxylation of acetyl/propionyl-CoA is proposed to proceed *via* binding of the CO_2-biotin to one of the two CS fold domains in a CT monomer. Polarization of the ureido carbonyl of CO_2-biotin by an OAH (formed by Gly182 and Gly183 in case of PCC from *Streptomyces coelicolor*) is proposed to induce loss of CO_2. Deprotonation of the α-carbon of acetyl/propionyl-CoA (by an active site base, proposed to be a cysteinyl residue in *E. coli* CT [79] or by the biotin N1 atom in yeast ACC CT (**XV**) [15] and PCC from *S. coelicolor* [16]) results in formation of an enolate stabilized by the OAH (in an adjacent CT monomer, formed by Gly419'-Ala420' in the case of PCC from *S. coelicolor*). The attack of the enolate on CO_2 generates malonyl- or methylmalonyl-CoA. The role of OAHs from different CT monomers in ACC catalysis exemplifies the importance of interactions between monomers for at least one of the CS.

The carboxyltransferase subunit of glutaconyl-CoA decarboxylase (Gcdα, VII)

The glutaconyl-CoA decarboxylase (Gcdα, **VII**) is a class 2 BDC that is a sodium ion pump protein. The energy released by the exothermic decarboxylation of glutaconyl-CoA to give crotonyl-CoA is proposed to drive ion transport from the cytoplasm into the

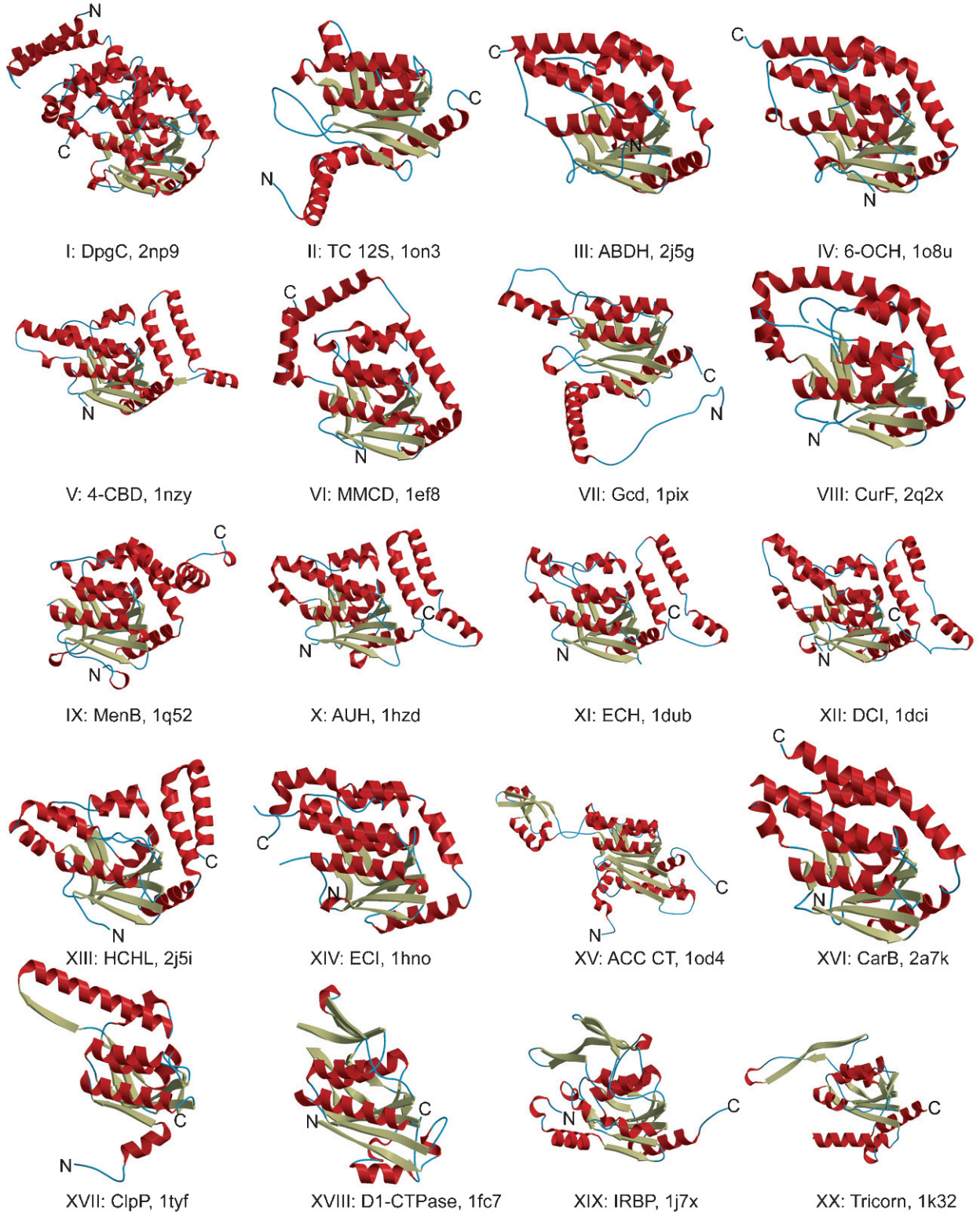


Figure 8. Overall folds of the monomers of the 20 enzymes considered in this review in the same order as in Table 1. For each structure, the assigned abbreviated name is shown and the PDB code given. For **II**, **VII** and **XV**, only the N-terminal of the two CS fold containing domains is shown. For **XVII**-**XX**, only the CS fold region of the monomer is shown. The cartoon representations are colored red for helices, khaki for sheets and lightblue for loops. For the full name of each enzyme and the corresponding reference, see Table 1. Some N- or C-terminal labels cannot be seen because of the orientation of the monomers. Figures were generated using Molscript [131] and Raster3D [132].

periplasm [13]. The crystal structure of Gcdα (**VII**) from *Acidaminococcus fermentans* bound to glutaconyl-CoA reveals Gcdα (**VII**) as a dimer with each monomer consisting of two CS fold domains [13]. Polarization of the thioester carbonyl of glutaconyl-CoA by one OAH, located in the N-terminal domain of one monomer, induces decarboxylation and dienolate formation. Direct transfer of the N1-proton of biotin, bound to the C-terminal domain of the other monomer, to the dienolate generates crotonyl-CoA. The resultant biotin anion attacks the CO_2 and generates N1-carboxybiotin [13, 86].

Transcarboxylase 12S (TC 12S, II)

The class 3 BDC transcarboxylase structure comprises three subunits: 12S, 5S and 1.3S. The monomer of the hexameric 12S consists of two CS fold domains (TC 12S, **II**). However, only the N-terminal domain of each 12S monomer binds ligand [27]. The overall TC transcarboxylation reaction consists of two stages [87–89]. In the first stage, 12S catalyzes carboxylate transfer from MMCoA to biotin attached to the 1.3S subunit. The second stage, catalyzed by the 5S domain (which is not a CS member), transfers the carboxylate from the 1.3S-biotin complex onto pyruvate.

In the 12S catalyzed first stage, it is proposed (on the basis of crystallographic analysis) that the thioester carbonyl group of MMCoA is polarized by a CS OAH formed by the back-bone nitrogen of Ala143 and, unusually, a water molecule that is hydrogen-bonded to the nitrogen of Ala183 and the carbonyl group of Ala180 [27]. If correct, this represents a highly unusual type of OAH. The MMCoA carboxylate is positioned to form hydrogen bonds to the backbone NHs of Gly182 (from one monomer) and Gly414' (from another monomer in the opposing trimer) as well as two water molecules. The mechanism of decarboxylation and carboxyl transfer to the 1.3S domain is unclear [27, 90].

CoA-independent CS members – 6-OCH (IV), ABDH (III), ClpP (XVII), D1CTPase (XVIII), IRBP (XIX) and Tricorn (XX)

The CS fold, sometimes with insertions or deletions, is also found in enzymes that do not employ CoA derivatives as substrates. Some of these enzymes contain an OAH homologous to those of the CoA substrate dependent CS members.

Monofunctional diketone hydrolases – 6-OCH (IV), ABDH (III)

6-Oxocamphor hydrolase (6-OCH, IV)

Camphor is a monoterpenoid of medicinal and commercial importance. 6-OCH (**IV**) catalyzes hydrolysis of the diketone 6-oxocamphor to α-campholinic acid, during camphor metabolism in *Rhodococcus* sp. NCIMB 9784. The 6-OCH (**IV**) reaction (Fig. 2) is stereospecific for reaction at the pro-(*S*)-carbon producing (4*S*)-, but not (4*R*)-, substituted products [91]. Camphor metabolism in *Rhodococcus* sp. first involves hydroxylation, catalyzed by a cytochrome p450 enzyme, followed by oxidation catalyzed by an alcohol dehydrogenase prior to the 6-OCH (**IV**) reaction [91]. The retro-Dieckmann condensation catalyzed by 6-OCH (**IV**) has a stereoselectivity of 6:1 in favor of (2*R*,4*S*)-α-campholinic acid [over (2*S*,4*S*)-] [92]. Other diketones can be hydrolyzed by 6-OCH (**IV**) including six-membered diketone rings bridged by two and three atom bridges, and a non-bridged cyclic diketone [93]. Unlike most CS members, 6-OCH (**IV**) does not employ a CoA thioester substrate nor possess an obvious OAH [94]. The first step in the reaction is proposed to be attack on the pro-(*S*)-carbonyl by a water molecule activated by His145 in conjunction with other residues [94, 95]. The resulting tetrahedral intermediate undergoes C-C fragmentation forming a carboxylic acid and an enolate anion. The enolate anion is proposed to be stabilized by hydrogen bonding interactions with either or both of the side chains of His121 or Trp40 [95]. The enolate derived from 6-oxocamphor can then protonate preferentially on the face opposite to the carboxylic acid to produce the less stable (2*S*,4*S*)-*trans* product, which tautomerizes upon release from the active site to yield the observed 6:1 (2*R*:2*S*) ratio [93, 95].

***Anabaena* β-diketone hydrolase (ABDH, III)**

ABDH (**III**) catalyzes the hydrolysis of bicycle[2.2.2]octane-2,6-dione to (*S*)-3-[oxocyclohexyl]-acetic acid [96] (Fig. 2). β-Diketones occur in the microbial metabolism of aromatics [97]. ABDH (**III**) catalyzes the same CoA-independent retro-Dieckmann condensation type reaction as 6-oxocamphor hydrolase (6-OCH, **IV**), but is reported to have a more limited range of substrates and a lower efficiency [96]. Like 6-OCH (**IV**), the first step of the ABDH (**III**) desymmetrization reaction is histidyl (His144) activated attack of a water onto the pro-(*S*)-carbonyl group to form a tetrahedral intermediate [96]. The tetrahedral intermediate formed is proposed to be stabilized by hydrogen bonding interactions with the side chain of His121, possibly as in 6-OCH (**IV**).

Active site differences may account for the difference in activity between 6-OCH (**IV**) and ABDH (**III**); Trp40 is proposed to stabilize the enolate formed and assist substrate orientation in the active site of 6-OCH (**IV**) but is substituted by Phe38 in ABDH (**III**), and Phe82, thought to be involved in stereocontrol in 6-OCH (**IV**), is substituted by Leu80 in ABDH (**III**) [96].

Other CoA independent enzymes/carrier proteins having the CS fold as part of their overall structure – ClpP (XVII), D1CTPase (XVIII), IRBP (XIX) and the Tricorn protease (XX)[1]

The proteolytic subunit of caseinolytic protease (ClpP, XVII)

Caseinolytic proteases (Clp) are ubiquitous and involved in various cellular activities, including degradation of misfolded proteins, regulation of short-lived proteins, and the housekeeping removal of dysfunctional proteins [98–103]. Clp is implicated in the control of cell growth and the regulation of virulence genes in the pathogens *Listeria monocytogenes* and *Salmonella typhimurium* [104–106].

Clp has a serine protease component, ClpP (**XVII**), and an ATPase component, ClpA (or ClpX in *E. coli*) [3, 4]. Superimposition of the monomers of ClpP (**XVII**) and enoyl-CoA hydratase (**XI**) gives a root mean square deviation (RMSD) (Cα atoms) of 2.3 Å across 137 aligned residues [107] and reveals conservation of the location of the OAH forming residues (Fig. 5B).

Crystal structures of ClpPs from four species have been reported: from *E. coli* (**XVII**) [108], *Homo sapiens* [109], *Streptococcus pneumoniae* [110] and *Mycobacterium tuberculosis* [111]. In addition, the structure of the *Plasmodium falciparum* ClpP has been deposited in the Protein Data Bank (PDB) (PDB code 2f6i).

The *E. coli* ClpP (**XVII**) is a tetradecamer comprising two heptamers stacked on top of each other forming a central cavity. The ClpP (**XVII**) monomer, which does not have the helical C-terminal subdomain observed in other CS members, binds to an adjacent monomer using a protruding α/β unit [108]. Inside the proteolytic chamber, each monomer contributes an active site catalytic triad (Ser97, His122 and Asp171). The side chain of Ser97 points towards the OAH defined by the amido groups of the conserved Gly68 and Met98. Substrates are unfolded with the aid of the associated ClpA and translocated through a channel into the proteolytic cavity of ClpP [112, 113]. The ATPase component acts as a molecular chaperone that can catalyze the unfolding of stable proteins [114].

Photosystem II D1 C-terminal processing protease (D1 CTPase, XVIII)

The photosystem II D1 C-terminal processing protease (D1 CTPase, **XVIII**), a serine protease, is involved in the continual renewal of the photosystem II D1 protein. The D1 CTPase (**XVIII**) catalyzes C-terminal proteolysis to give a functional D1 protein [115]. The D1 CTPase (**XVIII**) from *Scenedesmus obliquus* is monomeric and comprises three domains: (a) a helix bundle, (b) a PDZ-like domain [116] and (c) a catalytic domain. Domain C has a CS fold. The active site lies at the interface of the three domains. Domain B is proposed to be the site at which the D1 C-terminus binds. The remainder of the substrate is proposed to extend across the face of the D1 CTPase (**XVIII**), interacting at its scissile bond with a Ser372/Lys397 catalytic dyad in domain C. The active site is at the interface of the three domains [117]. Superimposition of the monomers of D1 CTPase (**XVIII**) and enoyl-CoA hydratase (**XI**) gives a RMSD (Cα atoms) of 2.7 Å across 132 residues [107] and reveals conservation of the OAH (Gly 318 and Ala 373 for D1 CTPase, **XVIII**).

Functional unit of interphotoreceptor retinoid binding protein (IRBP, XIX)

The interphotoreceptor retinoid binding protein (IRBP, **XIX)** is critical to the function, integrity and development of the vertebrate retina. The molecular role of IRBP (**XIX**) is uncertain, but it is proposed to protect 11-*cis*-retinal and all *trans*-retinol from isomeric and oxidative degradation, while facilitating their exchange between the photoreceptors and retinal-pigmented epithelium [118].

Mammalian, avian, and amphibian IRBPs consist of four functional units or modules. X2IRBP (module 2 of *Xenopus laevis* IRBP, **XIX**) is monomeric and consists of two domains separated by a hydrophobic ligand-binding site. The N-terminal domain forms a three-helix bundle followed by a small β-strand. The C-terminal domain has a CS fold with six β-strands surrounded by five α-helices [118] {RMSD of CS fold containing domain of IRBP (**XIX**) and ECH (**XI**) over 134 residues: 3.6 Å [107]}. Neither the ligand-binding site nor the OAH of X2IRBP has been identified [118]. The role of the CS fold in IRBP (**XIX**) is unknown.

[1] The relationship of the four proteins described in this section to the rest of the CS has not been extensively discussed. Structural alignments with ECH (**XI**) were carried out.

Tricorn protease (Tricorn, XX)

The tricorn serine protease (**XX**), from *Thermoplasma acidophilum*, is hexameric and can assemble into a giant icosahedral capsid proposed to serve as the organizing center of a multi-proteolytic complex [119]. The tricorn protease (**XX**) acts downstream of the proteasome to convert peptides generated by the proteasome into free amino acids [120]. The tricorn protease monomer has five domains, two open β-propeller structures of six and seven blades (Met39–-Asp310 and Ala326–Lys675), a helical bundle (Val679–Ser745, C1), a PDZ domain [116] (Arg761–Asp855) and CS fold domain (Arg856–Asn1061, C2) [121] {RMSD of CS fold containing domain of tricorn (**XX**) and ECH (**XI**) over 102 residues: 3.2 Å [107]}. The OAH is formed by the backbone NHs of Gly918 and Asp966.

As for the other CS proteases, the mechanism of the tricorn protease (**XX**) is proposed to be analogous to that of the serine proteases (Fig. 4) with Ser965 and His746 acting as a catalytic dyad [121].

Other CS members of interest for which structures have not been reported

2-Ketocyclohexanecarboxyl-CoA hydrolase, BadI

2-Ketocyclohexanecarboxyl-CoA (BadI) hydrolase catalyzes a reaction in the pathway for anaerobic degradation of benzoate in *Rhodopseudomonas palustris*. BadI catalyzes a retro-Dieckmann cyclization in which (2*S*)-ketocyclohexanecarboxyl-CoA is hydrolyzed to pimelyl-CoA (Figs. 2 and 4) and, in mechanistic terms, is similar to MenB (**IX**) [122].

Benzoyl-CoA-dihydrodiol lyase, BoxC

Benzoyl-CoA-dihydrodiol lyase (BoxC) catalyzes the ring cleavage of 2,3-dihydro-2,3-dihydroxybenzoyl-CoA into 3,4-dehydroadipyl-CoA semialdehyde (Figs. 2 and 4). Purified BoxC does not require divalent metals, molecular oxygen or any cosubstrates or coenzymes for activity. The BoxC reaction is part of a widely distributed process of aerobic aromatic metabolism in which the intermediates are coenzyme A thioesters and the ring-cleavage reactions do not require molecular oxygen [123].

Crotonobetainyl-CoA hydratase, CaiD

The reversible biotransformation of crotonobetaine to L-carnitine in *E. coli* proceeds *via* two steps: the hydration of crotonobetainyl-CoA to L-carnitinyl-CoA catalyzed by CS member CaiD (Fig. 2), followed by a CoA transfer from L-carnitinyl-CoA to crotonobetaine, catalyzed by crotonobetainyl-CoA: carnitine CoA-transferase (CaiB). CaiD can also hydrate crotonyl-CoA at a slower rate than crotonobetainyl-CoA but cannot hydrate crotonobetaine [124].

3-Hydroxyisobutyryl-CoA hydrolase

The mitochondrial enzyme 3-hydroxyisobutyryl-CoA (HIB-CoA) hydrolase (3-hydroxy-2-methylpropanoyl-CoA hydrolase, EC 3.1.2.4) catalyzes the hydrolysis of (*S*)-HIB-CoA, an intermediate in the pathway of valine catabolism. Hydrolysis of HIB-CoA (Fig. 2) may be an important strategy to protect cells against toxic effects of methacrylyl-CoA, an intermediate in the valine pathway occurring immediately upstream of HIB-CoA, which is an alkylating agent [125–127].

Conclusions

In recent years, significant advances have been made in defining the structures of many CS proteins. For some members, including the enoyl-CoA hydratases and isomerases, detailed mechanistic proposals are being developed. To develop a better understanding of catalysis and how selectivity is achieved by the CS, further information is required in particular on the structures of enzyme substrate/intermediate complexes, on the conformational changes during catalysis and on the precise mechanism of the deprotonation/protonation steps.

Given that CS enzymes can catalyze formation of ring-structures, carbon-carbon and carbon-heteroatom bonds, they would seem to have significant potential in biocatalysis/fermentation procedures for the production of valuable chemicals. The highly conserved nature of the OAH together with the 'modular' oligomerization structure of the CS suggests that they may be amenable to productive variation, possibly involving directed evolution or combinatorial approaches, *e.g.*, involving domain swapping, to generate new activities. Most of the classic CS enzymes [*e.g.*, ECH (**XI**), ECI (**XIV**) and DCI (**XII**)] appear to have a limited number of polar residues in their active sites. Thus, rational active site modifications, *e.g.*, to modify an α,β-hydratase to give a β,γ-hydratase or to alter the stereoselectivity of hydration may be possible.

To date, the limited number of reported mutagenesis studies on the CS have been focused on mechanistic studies, for example to trap intermediates/products for crystallographic analyses [11, 95]. Mutagenesis has also been used to modify ECI (**XIV**) [128] and 4-CBD (**V**) [26] such that they catalyze a hydratase [*i.e.*, ECH (**XI**)] reaction, demonstrating the viability of mutagenesis to obtain alternative function. The use of non coenzyme A substrate utilizing CS enzymes in biocatalysis has already been pioneered by Grogan et al.

[92] who employed 6-OCH (**IV**) for the desymmetrization of a number of bicyclic prochiral β-diketones. 6-OCH (**IV**) displayed good stereoselectivity and provided methodology for the preparation of useful chiral intermediates.

The closely related and often conserved structural features of CS members allied to the range of reactions that they catalyze suggests there may be an exciting future for the CS, particularly in catalyzing commercially important reactions.

Acknowledgement. We thank the BBSRC and the Ministry of higher education, Egypt for the financial support as well as all our laboratory members who helped with this work.

1 Stern, J. R. and del Campillo, A. (1953) Enzymatic reaction of crotonyl coenzyme A. J. Am. Chem. Soc. 75: 2277–2278.
2 Holden, H. M., Benning, M. M., Haller, T. and Gerlt, J. A. (2001) The crotonase superfamily: Divergently related enzymes that catalyze different reactions involving acyl coenzyme A thioesters. Acc. Chem. Res. 34: 145–157.
3 Gerlt, J. A. and Babbitt, P. C. (2001) Divergent evolution of enzymatic function: Mechanistically diverse superfamilies and functionally distinct suprafamilies. Annu. Rev. Biochem. 70: 209–246.
4 Murzin, A. G. (1998) How far divergent evolution goes in proteins. Curr. Opin. Struct. Biol. 8: 380–387.
5 Widboom, P. F., Fielding, E. N., Liu, Y. and Bruner, S. D. (2007) Structural basis for cofactor-independent dioxygenation in vancomycin biosynthesis. Nature 447: 342–345.
6 Sleeman, M. C. and Schofield, C. J. (2004) Carboxymethylproline synthase (CarB), an unusual carbon-carbon bond-forming enzyme of the crotonase superfamily involved in carbapenem biosynthesis. J. Biol. Chem. 279: 6730–6736.
7 Gerratana, B., Arnett, S. O., Stapon, A. and Townsend, C. A. (2004) Carboxymethylproline synthase from *Pectobacterium carotorova:* A multifaceted member of the crotonase superfamily. Biochemistry 43: 15936–15945.
8 Murzin, A. G., Brenner, S. E., Hubbard, T. and Chothia, C. (1995) SCOP: A structural classification of proteins database for the investigation of sequences and structures. J. Mol. Biol. 247: 536–540.
9 Mursula, A. M., Hiltunen, J. K. and Wierenga, R. K. (2004) Structural studies on Δ^3,Δ^2-enoyl-CoA isomerase: The variable mode of assembly of the trimeric disks of the crotonase superfamily. FEBS Lett. 557: 81–87.
10 Partanen, S. T., Novikov, D. K., Popov, A. N., Mursula, A. M., Kalervo Hiltunen, J. and Wierenga, R. K. (2004) The 1.3 Å crystal structure of human mitochondrial Δ^3,Δ^2-enoyl-CoA isomerase shows a novel mode of binding for the fatty acyl group. J. Mol. Biol. 342: 1197–1208.
11 Benning, M. M., Taylor, K. L., Liu, R. Q., Yang, G., Xiang, H., Wesenberg, G., Dunaway-Mariano, D. and Holden, H. M. (1996) Structure of 4-chlorobenzoyl coenzyme A dehalogenase determined to 1.8 Å resolution: An enzyme catalyst generated *via* adaptive mutation. Biochemistry 35: 8103–8109.
12 Sleeman, M. C., Sorensen, J. L., Batchelar, E. T., McDonough, M. A. and Schofield, C. J. (2005) Structural and mechanistic studies on carboxymethylproline synthase (CarB), a unique member of the crotonase superfamily catalyzing the first step in carbapenem biosynthesis. J. Biol. Chem. 280: 34956–34965.
13 Wendt, K. S., Schall, I., Huber, R., Buckel, W. and Jacob, U. (2003) Crystal structure of the carboxyltransferase subunit of the bacterial sodium ion pump glutaconyl-coenzyme A decarboxylase. EMBO J. 22: 3493–3502.
14 Bilder, P., Lightle, S., Bainbridge, G., Ohren, J., Finzel, B., Sun, F., Holley, S., Al-Kassim, L., Spessard, C., Melnick, M., Newcomer, M. and Waldrop, G. L. (2006) The structure of the carboxyltransferase component of acetyl-CoA carboxylase reveals a zinc-binding motif unique to the bacterial enzyme. Biochemistry 45: 1712–1722.
15 Zhang, H., Yang, Z., Shen, Y. and Tong, L. (2003) Crystal structure of the carboxyltransferase domain of acetyl-coenzyme A carboxylase. Science 299: 2064–2067.
16 Diacovich, L., Mitchell, D. L., Pham, H., Gago, G., Melgar, M. M., Khosla, C., Gramajo, H. and Tsai, S. C. (2004) Crystal structure of the β-subunit of acyl-CoA carboxylase: Structure-based engineering of substrate specificity. Biochemistry 43: 14027–14036.
17 Benning, M. M., Haller, T., Gerlt, J. A. and Holden, H. M. (2000) New reactions in the crotonase superfamily: Structure of methylmalonyl CoA decarboxylase from *Escherichia coli.* Biochemistry 39: 4630–4639.
18 Bennett, M. J., Schlunegger, M. P. and Eisenberg, D. (1995) 3D domain swapping: A mechanism for oligomer assembly. Protein Sci. 4: 2455–2468.
19 Hubbard, P. A., Yu, W., Schulz, H. and Kim, J. J. P. (2005) Domain swapping in the low-similarity isomerase/hydratase superfamily: The crystal structure of rat mitochondrial Δ^3,Δ^2-enoyl-CoA isomerase. Protein Sci. 14: 1545–1555.
20 Truglio, J. J., Theis, K., Feng, Y., Gajda, R., Machutta, C., Tonge, P. J. and Kisker, C. (2003) Crystal structure of *Mycobacterium tuberculosis* MenB, a key enzyme in vitamin K2 biosynthesis. J. Biol. Chem. 278: 42352–42360.
21 Hedstrom, L. (2002) Serine protease mechanism and specificity. Chem. Rev. 102: 4501–4523.
22 Otto, H. H. and Schirmeister, T. (1997) Cysteine proteases and their inhibitors. Chem. Rev. 97: 133–172.
23 Engel, C. K., Kiema, T. R., Kalervo Hiltunen, J. and Wierenga, R. K. (1998) The crystal structure of enoyl-CoA hydratase complexed with octanoyl-CoA reveals the structural adaptations required for binding of a long chain fatty acid-CoA molecule. J. Mol. Biol. 275: 847–859.
24 Li, J. J. and Bugg, T. D. H. (2007) Investigation of a general base mechanism for ester hydrolysis in C–C hydrolase enzymes of the α/β-hydrolase superfamily: A novel mechanism for the serine catalytic triad. Org. Biomol. Chem. 507–513.
25 Todd, A. E., Orengo, C. A. and Thornton, J. M. (2002) Plasticity of enzyme active sites. Trends Biochem. Sci. 27: 419–426.
26 Xiang, H., Luo, L., Taylor, K. L. and Dunaway-Mariano, D. (1999) Interchange of catalytic activity within the 2-enoyl-coenzyme A hydratase/isomerase superfamily based on a common active site template. Biochemistry 38: 7638–7652.
27 Hall, P. R., Wang, Y. F., Rivera-Hainaj, R. E., Zheng, X., Pustai-Carey, M., Carey, P. R. and Yee, V. C. (2003) Transcarboxylase 12S crystal structure: Hexamer assembly and substrate binding to a multienzyme core. EMBO J. 22: 2334–2347.
28 Waterson, R. M., Hass, G. M. and Hill, R. L. (1972) Enoyl-coenzyme A hydratase (crotonase). Enhancement of the rate of hydration of crotonylpantetheine by coenzyme A and related compounds. J. Biol. Chem. 247: 5252–5257.
29 Person, N. B. (1981) State University of New York at Buffalo, Buffalo, NY.
30 Willadsen, P. and Eggerer, H. (1975) Substrate stereochemistry of the enoyl-CoA hydratase reaction. Eur. J. Biochem. 54: 247–252.
31 Fukui, T., Shiomi, N. and Doi, Y. (1998) Expression and characterization of (*R*)-specific enoyl-coenzyme A hydratase involved in polyhydroxyalkanoate biosynthesis by *Aeromonas caviae.* J. Bacteriol. 180: 667–673.
32 Engel, C. K., Mathieu, M., Zeelen, J. P., Hiltunen, J. K. and Wierenga, R. K. (1996) Crystal structure of enoyl-coenzyme A (CoA) hydratase at 2.5 Å resolution: A spiral fold defines the CoA-binding pocket. EMBO J. 15: 5135–5145.

33 Muller-Newen, G., Jansen, U. and Stoffel, W. (1995) Enoyl-CoA hydratase and isomerase form a superfamily with a common active-site glutamate residue. Eur. J. Biochem. 228: 68–73.

34 Feng, Y., Hofstein, H. A., Zwahlen, J. and Tonge, P. J. (2002) Effect of mutagenesis on the stereochemistry of enoyl-CoA hydratase. Biochemistry 41: 12883–12890.

35 Bahnson, B. J. and Anderson, V. E. (1991) Crotonase catalysed β-elimination is concerted- a double isotope effect study. Biochemistry 30: 5894–5906.

36 Bahnson, B. J., Anderson, V. E. and Petsko, G. A. (2002) Structural mechanism of enoyl-CoA hydratase: Three atoms from a single water are added in either an E1cb stepwise or concerted fashion. Biochemistry 41: 2621–2629.

37 Kiema, T. R., Engel, C. K., Schmitz, W., Filppula, S. A., Wierenga, R. K. and Hiltunen, J. K. (1999) Mutagenic and enzymological studies of the hydratase and isomerase activities of 2-enoyl-CoA hydratase-1. Biochemistry 38: 2991–2999.

38 Agnihotri, G. and Liu, H. W. (2003) Enoyl-CoA hydratase: Reaction, mechanism, and inhibition. Bioorg. Med. Chem. 11: 9–20.

39 Hiltunen, J. K. and Qin, Y. M. (2000) β-Oxidation – Strategies for the metabolism of a wide variety of acyl-CoA esters. Biochim. Biophys. Acta 1484: 117–128.

40 Mursula, A. M., van Aalten, D. M. F., Hiltunen, J. K. and Wierenga, R. K. (2001) The crystal structure of Δ^3,Δ^2-enoyl-CoA isomerase. J. Mol. Biol. 309: 845–853.

41 Geisbrecht, B. V., Zhu, D., Schulz, K., Nau, K., Morrell, J. C., Geraghty, M., Schulz, H., Erdmann, R. and Gould, S. J. (1998) Molecular characterization of *Saccharomyces cerevisiae* Δ^3,Δ^2-enoyl-CoA isomerase. J. Biol. Chem. 273: 33184–33191.

42 Gurvitz, A., Mursula, A. M., Firzinger, A., Hamilton, B., Kilpelainen, S. H., Hartig, A., Ruis, H., Hiltunen, J. K. and Rottensteiner, H. (1998) Peroxisomal Δ^3-*cis*-Δ^2-*trans*-enoyl-CoA isomerase encoded by ECI1 is required for growth of the yeast *Saccharomyces cerevisiae* on unsaturated fatty acids. J. Biol. Chem. 273: 31366–31374.

43 Luo, M. J., Smeland, T. E., Shoukry, K. and Schulz, H. (1994) $\Delta^{3,5}$,$\Delta^{2,4}$-Dienoyl-CoA isomerase from rat liver mitochondria. J. Biol. Chem. 269: 2384–2388.

44 Modis, Y., Filppula, S. A., Novikov, D. K., Norledge, B., Kalervo Hiltunen, J. and Wierenga, R. K. (1998) The crystal structure of dienoyl-CoA isomerase at 1.5 Å resolution reveals the importance of aspartate and glutamate sidechains for catalysis. Structure 6: 957–970.

45 Muller, R., Thiele, J., Klages, U. and Lingens, F. (1984) Incorporation of [^{18}O]water into 4-hydroxybenzoic acid in the reaction of 4-chlorobenzoate dehalogenase from *Pseudomonas* sp. strain CBS 3. Biochem. Biophys. Res. Commun. 124: 178–182.

46 Liu, R.-Q., Liang, P.-H., Scholten, J. and Dunaway-Mariano, D. (1995) Transient state kinetic analysis of the chemical intermediates formed in the enzymatic dehalogenation of 4-chlorobenzoyl coenzyme A. J. Am. Chem. Soc. 117: 5003–5004.

47 Yang, G., Liang, P.-H. and Dunaway-Mariano, D. (1994) Evidence for nucleophilic catalysis in the aromatic substitution reaction catalyzed by (4-chlorobenzoyl)coenzyme A dehalogenase. Biochemistry 33: 8527–8531.

48 Taylor, K. L., Xiang, H., Liu, R. Q., Yang, G. and Dunaway-Mariano, D. (1997) Investigation of substrate activation by 4-chlorobenzoyl-coenzyme A dehalogenase. Biochemistry 36: 1349–1361.

49 Tseng, C. C., Vaillancourt, F. H., Bruner, S. D. and Walsh, C. T. (2004) DpgC is a metal and cofactor-free 3,5-dihydroxyphenylacetyl-CoA 1,2-dioxygenase in the vancomycin biosynthetic pathway. Chem. Biol. 11: 1195–1203.

50 Chen, H., Tseng, C. C., Hubbard, B. K. and Walsh, C. T. (2001) Glycopeptide antibiotic biosynthesis: Enzymatic assembly of the dedicated amino acid monomer (S)-3,5-dihydroxyphenylglycine. Proc. Natl. Acad. Sci. USA 98: 14901–14906.

51 Hubbard, B. K. and Walsh, C. T. (2003) Vancomycin assembly: Nature's way. Angew. Chem. Int. Ed. 42: 730–765.

52 Fielding, E. N., Widboom, P. F. and Bruner, S. D. (2007) Substrate recognition and catalysis by the cofactor-independent dioxygenase DpgC. Biochemistry 46: 13994–14000.

53 Meganathan, R. and Bentley, R. (1981) Synthesis of *ortho*-succinylbenzoic acid in a Men *Escherichia coli* mutant requires decarboxylation of L-glutamate at the C-1 position. Biochemistry 20: 5336–5340.

54 Meganathan, R., Bentley, R. and Taber, H. (1981) Identification of *Bacillus subtilis* Men mutants which lack *ortho*-succinylbenzoyl CoA synthetase and dihydroxynapthoate synthase. J. Bacteriol. 145: 328–332.

55 Sharma, V., Suvarna, K., Meganathan, R. and Hudspeth, M. E. (1992) Menaquinone (Vitamin-K2) biosynthesis nucleotide sequence and expression of the MenB gene from *Escherichia coli.* J. Bacteriol. 174: 5057–5062.

56 Gasson, M. J., Kitamura, Y., McLauchlan, W. R., Narbad, A., Parr, A. J., Lindsay, E., Parsons, H., Payne, J., Rhodes, M. J. C. and Walton, N. J. (1998) Metabolism of ferulic acid to vanillin – A bacterial gene of the enoyl-SCoA hydratase/isomerase superfamily encodes an enzyme for the hydration and cleavage of a hydroxycinnamic acid SCoA thioester. J. Biol. Chem. 273: 4163–4170.

57 Leonard, P. M., Brzozowski, A. M., Lebedev, A., Marshall, C. M., Smith, D. J., Verma, C. S., Walton, N. J. and Grogan, G. (2006) The 1.8 Å resolution structure of hydroxycinnamoyl-coenzyme A hydratase-lyase (HCHL) from *Pseudomonas fluorescens*, an enzyme that catalyses the transformation of feruloyl-coenzyme A to vanillin. Acta Crystallogr. D Biol. Crystallogr. 62: 1494–1501.

58 Nakagawa, J., Waldner, H., Meyer-Monard, S., Hofsteenge, J., Jeno, P. and Moroni, C. (1995) AUH, a gene encoding an AU-specific RNA binding protein with intrinsic enoyl-CoA hydratase activity. Proc. Natl. Acad. Sci. USA 92: 2051–2055.

59 Wong, B. J. and Gerlt, J. A. (2004) Evolution of function in the crotonase superfamily: (3*S*)-Methylglutaconyl-CoA hydratase from *Pseudomonas putida.* Biochemistry 43: 4646–4654.

60 Mack, M., Liesert, M., Zschocke, J., Peters, V., Linder, D. and Buckel, W. (2006) 3-Methylglutaconyl-CoA hydratase from *Acinetobacter* sp. Arch. Microbiol. 185: 297–306.

61 Kurimoto, K., Fukai, S., Nureki, O., Muto, Y. and Yokoyama, S. (2001) Crystal structure of human AUH protein, a single-stranded RNA binding homolog of enoyl-CoA hydratase. Structure 9: 1253–1263.

62 Ijlst, L., Loupatty, F. J., Ruiter, J. P. N., Duran, M., Lehnert, W. and Wanders, R. J. A. (2002) 3-Methylglutaconic aciduria type I is caused by mutations in AUH. Am. J. Hum. Genet. 71: 1463–1466.

63 Ly, T. B., Peters, V., Gibson, K. M., Liesert, M., Buckel, W., Wilcken, B., Carpenter, K., Ensenauer, R., Hoffmann, G. F., Mack, M. and Zschocke, J. (2003) Mutations in the AUH gene cause 3-methylglutaconic aciduria type I. Hum. Mutat. 21: 401–407.

64 Rodriguez, J. M., Ruiz-Sala, P., Ugarte, M. and Penalva, M. A. (2004) Fungal metabolic model for type I 3-methylglutaconic aciduria. J. Biol. Chem. 279: 32385–32392.

65 Bearne, S. L. and Wolfendon, R. (1995) Glutamate γ-semialdehyde as a natural transition state analogue inhibitor of *Escherichia coli* glucosamine-6-phosphate synthase. Biochemistry 34: 11515–11520.

66 Wong, B. J. and Gerlt, J. A. (2003) Divergent function in the crotonase superfamily: An anhydride intermediate in the reaction catalyzed by 3-hydroxyisobutyryl-CoA hydrolase. J. Am. Chem. Soc. 125: 12076–12077.

67 Nunez, L. E., Mendez, C., Brana, A. F., Blanco, G., Salas, J. A. (2003) The biosynthetic gene cluster for the β-lactam carbapenem thienamycin in *Streptomyces cattleya.* Chem. Biol. 10: 301–311.

68 Galivan, J. H. and Allen, S. H. G. (1968) Methylmalonyl coenzyme A decarboxylase. Its role in succinate decarboxylation by *Micrococcus lactilyticus.* J. Biol. Chem. 243: 1253–1261.

69 Geders, T. W., Gu, L., Mowers, J. C., Liu, H., Gerwick, W. H., Hakansson, K., Sherman, D. H. and Smith, J. L. (2007) Crystal structure of the ECH2 catalytic domain of CurF from *Lyngbya majuscula:* Insights into a decarboxylase involved in polyketide chain β-branching. J. Biol. Chem. 282: 35954-35963.

70 Gerwick, W. H., Proteau, P. J., Nagle, D. G., Hamel, E., Blokhin, A. and Slate, D. L. (1994) Structure of curacin A, a novel antimitotic, antiproliferative and brine shrimp toxic natural product from the marine cyanobacterium *Lyngbya majuscula.* J. Org. Chem. 59: 1243–1245.

71 Calderone, C. T., Iwig, D. F., Dorrestein, P. C., Kelleher, N. L. and Walsh, C. T. (2007) Incorporation of nonmethyl branches by isoprenoid-like logic: Multiple β-alkylation events in the biosynthesis of Myxovirescin A1. Chem. Biol. 14: 835–846.

72 Butcher, R. A., Schroeder, F. C., Fischbach, M. A., Straight, P. D., Kolter, R., Walsh, C. T. and Clardy, J. (2007) The identification of bacillaene, the product of the PksX megacomplex in *Bacillus subtilis.* Proc. Natl. Acad. Sci. USA 104: 1506.

73 Pulsawat, N., Kitani, S. and Nihira, T. (2007) Characterization of biosynthetic gene cluster for the production of virginiamycin M, a streptogramin type A antibiotic, in *Streptomyces virginiae.* Gene 393: 31–42.

74 Simunovic, V. and Muller, R. (2007) 3-hydroxy-3-methylglutaryl-CoA-like synthases direct the formation of methyl and ethyl side groups in the biosynthesis of the antibiotic myxovirescin A. ChemBioChem 8: 497–500.

75 El-Sayed, A. K., Hothersall, J., Cooper, S. M., Stephens, E., Simpson, T. J. and Thomas, C. M. (2003) Characterization of the mupirocin biosynthesis gene cluster from *Pseudomonas fluorescens* NCIMB 10586. Chem. Biol. 10: 419–430.

76 Edwards, D. J., Marquez, B. L., Nogle, L. M., McPhail, K., Goeger, D. E., Roberts, M. A. and Gerwick, W. H. (2004) Structure and biosynthesis of the jamaicamides, new mixed polyketide-peptide neurotoxins from the marine cyanobacterium *Lyngbya majuscula.* Chem. Biol. 11: 817–833.

77 Piel, J., Butzke, D., Fusetani, N., Hui, D., Platzer, M., Wen, G. and Matsunaga, S. (2005) Exploring the chemistry of uncultivated bacterial symbionts: Antitumor polyketides of the oederin family. J. Nat. Prod. 68: 472–479.

78 Yamada, M., Natsume, R., Nakamatsu, T., Horinouchi, S., Kawasaki, H. and Senda, T. (2007) Crystallization and preliminary crystallographic analysis of DtsR1, a carboxyltransferase subunit of acetyl-CoA carboxylase from *Corynebacterium glutamicum.* Acta Crystallogr. Sect. F Struct. Biol. Cryst. Commun. 63: 120–122.

79 Blanchard, C. Z. and Waldrop, G. L. (1998) Overexpression and kinetic characterization of the carboxyltransferase component of acetyl-CoA carboxylase. J. Biol. Chem. 273: 19140–19145.

80 Knowles, J. R. (1989) The mechanism of biotin-dependent enzymes. Annu. Rev. Biochem. 58: 195–221.

81 Samols, D., Thornton, C. G., Murtif, V. L., Kumar, G. K., Hase, F. C. and Wood, H. G. (1988) Evolutionary conservation among biotin enzymes. J. Biol. Chem. 263: 6461–6464.

82 Wood, H. G. and Barden, R. E. (1977) Biotin enzymes. Annu. Rev. Biochem. 46: 385–413.

83 Lin, T. W., Melgar, M. M., Kurth, D., Swamidass, S. J., Purdon, J., Tseng, T., Gago, G., Baldi, P., Gramajo, H. and Tsai, S. C. (2006) Structure-based inhibitor design of AccD5, an essential acyl-CoA carboxylase carboxyltransferase domain of *Mycobacterium tuberculosis.* Proc. Natl. Acad. Sci. USA 103: 3072–3077.

84 Cronan Jr, J. E. and Waldrop, G. L. (2002) Multi-subunit acetyl-CoA carboxylases. Prog. Lipid Res. 41: 407–435.

85 Diacovich, L., Peiru, S., Kurth, D., Rodriguez, E., Podesta, F., Khosla, C. and Gramajo, H. (2002) Kinetic and structural analysis of a new group of acyl-CoA carboxylases found in *Streptomyces coelicolor* A3(2). J. Biol. Chem. 277: 31228–31236.

86 Boiangiu, C. D., Jayamani, E., Brugel, D., Herrmann, G., Kim, J., Forzi, L., Hedderich, R., Vgenopoulou, I., Pierik, A. J. and Steuber, J. (2005) Sodium ion pumps and hydrogen production in glutamate fermenting anaerobic bacteria. J. Mol. Microbiol. Biotechnol. 10: 105–119.

87 Wood, H. G. and Zwolinski, G. K. (1976) Transcarboxylase: Role of biotin, metals, and subunits in the reaction and its quaternary structure. CRC Crit. Rev. Biochem. 4: 47–122.

88 Wood, H. G. (1979) The anatomy of transcarboxylase and the role of its subunits. CRC Crit. Rev. Biochem. 7: 143–160.

89 Wood, H. G. and Kumar, G. K. (1985) Transcarboxylase – Its quaternary structure and the role of the biotinyl subunit in the assembly of the enzyme and in catalysis. Ann. N. Y. Acad. Sci. 447: 1–22.

90 Carey, P. R., Sonnichsen, F. D. and Yee, V. C. (2004) Transcarboxylase: One of nature's early nanomachines. IUBMB Life 56: 575–583.

91 Chapman, P. J., Meerman, G., Gunsalus, I. C., Srinivasan, R. and Rinehart, K. L. Jr. (1966) A new acyclic acid metabolite in camphor oxidation. J. Am. Chem. Soc. 88: 618–619.

92 Grogan, G., Graf, J., Jones, A., Parsons, S., Turner, N. J. and Flitsch, S. L. (2001) An asymmetric enzyme-catalyzed retro-Claisen reaction for the desymmetrization of cyclic beta-diketones. Angew. Chem. Int. Ed. 113: 1111–1114.

93 Grogan, G., Roberts, G. A., Bougioukou, D., Turner, N. J. and Flitsch, S. L. (2001) The desymmetrization of bicyclic β-diketones by an enzymatic retro-Claisen reaction. A new reaction of the crotonase superfamily. J. Biol. Chem. 276: 12565–12572.

94 Whittingham, J. L., Turkenburg, J. P., Verma, C. S., Walsh, M. A. and Grogan, G. (2003) The 2-Å crystal structure of 6-oxo camphor hydrolase: New structural diversity in the crotonase superfamily. J. Biol. Chem. 278: 1744–1750.

95 Leonard, P. M. and Grogan, G. (2004) Structure of 6-oxo camphor hydrolase H122A mutant bound to its natural product, (2*S*,4*S*)-α-campholinic acid. Mutant structure suggests an atypical mode of transition state binding for a crotonase homolog. J. Biol. Chem. 279: 31312–31317.

96 Bennett, J. P., Whittingham, J. L., Brzozowski, A. M., Leonard, P. M. and Grogan, G. (2007) Structural characterization of a β-diketone hydrolase from the *Cyanobacterium anabaena* sp. PCC 7120 in native and product-bound forms, a coenzyme A-independent member of the crotonase superfamily. Biochemistry 46: 137–144.

97 Pokorny, D., Steiner, W. and Ribbons, D. W. (1997) β-Ketolases-forgotten hydrolytic enzymes? Trends Biotechnol. 15: 291–296.

98 Aizenman, E., Engelberg-Kulka, H. and Glaser, G. (1996) An *Escherichia coli* chromosomal "addiction module" regulated by 3′,5′-bispyrophosphate: A model for programmed bacterial cell death. Proc. Natl. Acad. Sci. USA 93: 6059–6063.

99 Frees, D. and Ingmer, H. (1999) ClpP participates in the degradation of misfolded protein in Lactococcus lactis. Mol. Microbiol. 31: 79–87.

100 Gerth, U., Krüger, E., Derre, I., Msadek, T. and Hecker, M. (1998) Stress induction of the *Bacillus subtilis* clpP gene encoding a homologue of the proteolytic component of the Clp protease and the involvement of ClpP and ClpX in stress tolerance. Mol. Microbiol. 28: 787–802.

101 Krüger, E., Witt, E., Ohlmeier, S., Hanschke, R. and Hecker, M. (2000) The Clp proteases of *Bacillus subtilis* are directly involved in degradation of misfolded proteins. J. Bacteriol. 182: 3259–3265.

102 Maurizi, M. R. (1992) Proteases and protein degradation in *Escherichia coli.* Experientia 48: 178–201.

103 Tomoyasu, T., Mogk, A., Langen, H., Goloubinoff, P. and Bukau, B. (2001) Genetic dissection of the roles of chaperones and proteases in protein folding and degradation in the *Escherichia coli* cytosol. Mol. Microbiol. 40: 397–413.

104 Gaillot, O., Pellegrini, E., Bregenholt, S., Nair, S. and Berche, P. (2000) The ClpP serine protease is essential for the intracellular parasitism and virulence of *Listeria monocytogenes.* Mol. Microbiol. 35: 1286–1294.
105 Stephani, K., Weichart, D. and Hengge, R. (2003) Dynamic control of Dps protein levels by ClpXP and ClpAP proteases in *Escherichia coli.* Mol. Microbiol. 49: 1605–1614.
106 Webb, C., Moreno, M., Wilmes-Riesenberg, M., Curtiss Iii, R. and Foster, J. W. (1999) Effects of DksA and ClpP protease on sigma S production and virulence in *Salmonella typhimurium.* Mol. Microbiol. 34: 112–123.
107 Holm, L. (2000) DaliLite workbench for protein structure comparison. Bioinformatics 16: 566.
108 Wang, J., Hartling, J. A. and Flanagan, J. M. (1997) The structure of ClpP at 2.3 Å resolution suggests a model for ATP-dependent proteolysis of outstanding interest. Cell 91: 447–456.
109 Kang, S. G., Dimitrova, M. N., Ortega, J., Ginsburg, A. and Maurizi, M. R. (2005) Human mitochondrial ClpP is a stable heptamer that assembles into a tetradecamer in the presence of ClpX. J. Biol. Chem. 280: 35424–35432.
110 Gribun, A., Kimber, M. S., Ching, R., Sprangers, R., Fiebig, K. M. and Houry, W. A. (2005) The ClpP double ring tetradecameric protease exhibits plastic ring-ring interactions, and the N termini of its subunits form flexible loops that are essential for ClpXP and ClpAP complex formation. J. Biol. Chem. 280: 16185–16196.
111 Ingvarsson, H., Mate, M. J., Hogbom, M., Portnoi, D., Benaroudj, N., Alzari, P. M., Ortiz-Lombardia, M. and Unge, T. (2007) Insights into the inter-ring plasticity of caseinolytic proteases from the X-ray structure of *Mycobacterium tuberculosis* ClpP1. Acta Crystallogr. D. Biol. Crystallogr. 63: 249–259.
112 Ortega, J., Lee, H. S., Maurizi, M. R. and Steven, A. C. (2002) Alternating translocation of protein substrates from both ends of ClpXP protease. EMBO J. 21: 4938–4949.
113 Schirmer, E. C., Glover, J. R., Singer, M. A. and Lindquist, S. (1996) HSP100/Clp proteins: A common mechanism explains diverse functions. Trends Biochem. Sci. 21: 289–296.
114 Singh, S. K., Grimaud, R., Hoskins, J. R., Wickner, S. and Maurizi, M. R. (2000) Unfolding and internalization of proteins by the ATP-dependent proteases ClpXP and ClpAP. Proc. Natl. Acad. Sci. USA 97: 8898–8903.
115 Shestakov, S. V., Anbudurai, P. R., Stanbekova, G. E., Gadzhiev, A., Lind, L. K. and Pakrasi, H. B. (1994) Molecular cloning and characterization of the ctpA gene encoding a carboxyl-terminal processing protease. Analysis of a spontaneous photosystem II-deficient mutant strain of the *Cyanobacterium synechocystis* sp. PCC 6803. J. Biol. Chem. 269: 19354–19359.
116 Doyle, D. A., Lee, A., Lewis, J., Kim, E., Sheng, M. and MacKinnon, R. (1996) Crystal structures of a complexed and peptide-free membrane protein-binding domain: Molecular basis of peptide recognition by PDZ. Cell 85: 1067–1076.
117 Liao, D. I., Qian, J., Chisholm, D. A., Jordan, D. B. and Diner, B. A. (2000) Crystal structures of the photosystem II D1 C-terminal processing protease. Nat. Struct. Biol. 7: 749–753.
118 Loew, A. and Gonzalez-Fernandez, F. (2002) Crystal structure of the functional unit of interphotoreceptor retinoid binding protein. Structure 10: 43–49.
119 Walz, J., Tamura, T., Tamura, N., Grimm, R., Baumeister, W. and Koster, A. J. (1997) Tricorn protease exists as an icosahedral supermolecule *in vivo.* Mol. Cell 1: 59–65.
120 Brandstetter, H., Kim, J. S., Groll, M., Göttig, P. and Huber, R. (2002) Structural basis for the processive protein degradation by tricorn protease. Biol. Chem. 383: 1157–1165.
121 Brandstetter, H., Kim, J.-S., Groll, M. and Huber, R. (2001) Crystal structure of the tricorn protease reveals a protein disassembly line. Nature 414: 466–470.
122 Eberhard, E. D. and Gerlt, J. A. (2004) Evolution of function in the crotonase superfamily: The stereochemical course of the reaction catalyzed by 2-ketocyclohexanecarboxyl-CoA hydrolase. J. Am. Chem. Soc. 126: 7188–7189.
123 Gescher, J., Eisenreich, W., Worth, J., Bacher, A. and Fuchs, G. (2005) Aerobic benzoyl-CoA catabolic pathway in *Azoarcus evansii:* Studies on the non-oxygenolytic ring cleavage enzyme. Mol. Microbiol. 56: 1586–1600.
124 Elssner, T., Engemann, C., Baumgart, K. and Kleber, H. P. (2001) Involvement of coenzyme A esters and two new enzymes, an enoyl-CoA hydratase and a CoA-transferase, in the hydration of crotonobetaine to L-carnitine by *Escherichia coli.* Biochemistry 40: 11140–11148.
125 Shimomura, Y., Murakami, T., Nakai, N., Huang, B. L., Hawes, J. W. and Harris, R. A. (2000) 3-hydroxyisobutyryl-CoA hydrolase. Methods Enzymol. 324: 229–240.
126 Shimomura, Y., Murakami, T., Fujitsuka, N., Nakai, N., Sato, Y., Sugiyama, S., Shimomura, N., Irwin, J., Hawes, J. W. and Harris, R. A. (1994) Purification and partial characterization of 3-hydroxyisobutyryl-coenzyme A hydrolase of rat liver. J. Biol. Chem. 269: 14248–14253.
127 Hawes, J. W., Jaskiewicz, J., Shimomura, Y., Huang, B. L., Bunting, J., Harper, E. T. and Harris, R. A. (1996) Primary structure and tissue-specific expression of human β-hydroxyisobutyryl-coenzyme A hydrolase. J. Biol. Chem. 271: 26430–26434.
128 Yu, W., Chu, X., Deng, G., Liu, X., Chen, G. and Li, D. (2006) Mutation of Lys242 allows Δ^3,Δ^2-enoyl-CoA isomerase to acquire enoyl-CoA hydratase activity. Biochim. Biophys. Acta 1760: 1874–1883.
129 Bond, C. S. (2003) TopDraw: A sketchpad for protein structure topology cartoons. Bioinformatics 19: 311–312.
130 DeLano, W. L. (2002) The PyMOL Molecular Graphics System. http://www. pymol. org DeLano Scientific LLC, San Carlos, CA, USA.
131 Kraulis, P. J. (1991) MOLSCRIPT: A program to produce both detailed and schematic plots of proteins. J. Appl. Crystallogr. 24: 946–950.
132 Merritt, E. A. and Bacon, D. J. (1997) Raster3D: Photorealistic molecular graphics. Methods Enzymol. 277: 505–524.
133 Krissinel, E. and Henrick, K. (2004) Secondary-structure matching (SSM), a new tool for fast protein structure alignment in three dimensions. Acta Crystallogr. D. Biol. Crystallogr. 60: 2256–2268.
134 Nicholas, K. B., Nicholas, H. B. and Deerfield, D. W. (1997) GeneDoc: Analysis and visualization of genetic variation. EMBNEW. NEWS 4: 14.