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Recombinant expression of a unique chloromuconolactone dehalogenase ClcF from *Rhodococcus opacus* 1CP and identification of catalytically relevant residues by mutational analysis

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ABSTRACT

Chloromuconolactone dehalogenase ClcF plays a unique role in 3-chlorocatechol degradation by *Rhodococcus opacus* 1CP by compensating the inability of its chloromuconate cycloisomerase ClcB2 to dechlorinate the chemically stable cycloisomerization product (4*R*,5*S*)-5-chloromuconolactone (5CML). High sequence similarities showed relatedness of ClcF to muconolactone isomerases (MLIs, EC 5.3.3.4) of the 3-oxoadipate pathway. Although both enzyme types share the ability to dechlorinate 5CML, comparison of *kcat/Km* indicated a significant extent of specialization of ClcF for dechlorination. This assumption was substantiated by an almost complete inability of ClcF to convert (4*S*)-muconolactone and the exclusive formation of *cis*-dienelactone from 5CML. Mutational analysis of ClcF by means of variants E27D, E27Q, Y50A, N52A, and A89S indicated relevance of some highly conserved residues for substrate binding and catalysis. Based on the putative isomerization mechanism of MLI, evidence was provided for a role of E27 in initial proton abstraction as well as of Y50 and N52 in substrate binding. In case of N52 substrate binding is likely to occur to the carboxylic group of 5CML as indicated by a significant change of product specificity. Expression in *Escherichia coli* BL21-CP(DE)-RIL followed by a three-step purification procedure with heat treatment is a convenient strategy to obtain recombinant ClcF and variants thereof.

Introduction

Dehalogenation mechanisms probably play the most critical role in the detoxification and mineralization of chloroorganic compounds by microorganisms. Organically bound chlorine results in an increase of persistence, toxicity, and tendency of bioaccumulation and thus is responsible for the high environmental relevance of this heterogeneous group of substances [1]. Incomplete elimination of chlorine may result in the formation of intermediates of even increased toxicity, as it is the case for the accumulation of mutagenic vinyl chloride during dehalorespiration of tetrachloroethene [2,3] or it may lead to an inhibition of degradation pathways not suited for dehalogenation [4,5].

Various dechlorination mechanisms have been identified in the aerobic degradation of chloroaromatic compounds. These either occur at an early point of peripheral degradation and/or at a late stage in the central catabolism of chlorocatechol intermediates. The modified *ortho*-cleavage pathway is probably the most common route for the aerobic degradation of chlorocatechols and has been described in detail for representatives of the phylum Proteobacteria [6,7].

Investigations into the biochemistry and genetics of chloroaromatic degradation in the actinobacterium *Rhodococcus opacus* 1CP recently pointed to an alternative type of modified *ortho*-cleavage pathway which obviously evolved independently from that found in Proteobacteria by functionally convergent evolution [8–10]. In addition to a low phylogenetic relatedness, the chloromuconate cycloisomerases of strain 1CP and Proteobacteria fundamentally differ in their ability to dechlorinate. While proteobacterial chloromuconate cycloisomerases lactonize and dechlorinate 2-chloro-*cis*,*cis*-muconate (an intermediate of 3-chlorocatechol catabolism) into *trans*-dienelactone ((2*Z*)-2-(5-oxo-2(5*H*)-furanylidene)acetic acid) (Fig. 1A), the corresponding enzyme ClcB2 of strain 1CP only catalyzes lactonization to (4*R*,5*S*)-5-chloromuconolatone[1] (5CML) as a stable intermediate without further dehalogenation (Fig. 1B) [11]. The inability of ClcB2 to dechlorinate is shared by muconate cycloisomerases of Proteobacteria and Actinobacteria [8,12], a fact consistent the latter type of enzyme representing the putative ances-

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[1] *Abbreviations used*: 5CML, 5-chloromuconolactone; ELH, 3-oxoadipate enol-lactone hydrolase; MLI, muconolactone isomerase, IPTG, isopropyl-β-D-thiogalacto-pyranoside; SDS, sodium dodecyl sulfate.

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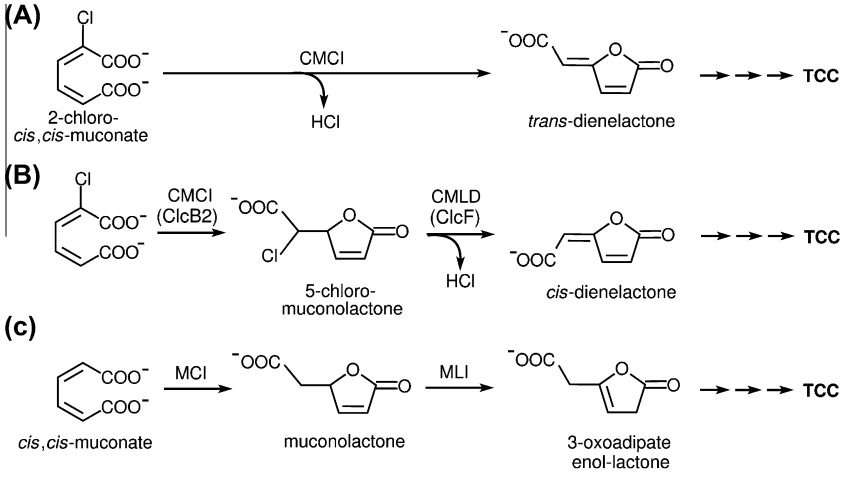

Fig. 1. (A) Function of proteobacterial chloromuconate cycloisomerases CMCI in 3-chlorocatechol degradation, (B) the ClcF-comprising variant of this pathway in *R. opacus* 1CP, and (C) the physiological role of muconolactone isomerases in catechol degradation. The reactions above represent only an excerpt of the corresponding pathways. CMCI, chloromuconate cycloisomerase; CMLD, 5-chloromuconolactone dehalogenase; MCI, muconate cycloisomerase; MLI, muconolactone isomerase. Abbreviations for the gene product in strain 1CP are given in parenthesis.

tor. In strain 1CP dechlorination of 5CML is achieved by a hitherto unique type of 5-chloromuconolactone dehalogenase, encoded by gene *clcF*, to yield *cis*-dienelactone ((2*E*)-2-(5-oxo-2(5*H*)-furanylidene)acetic acid) [10].

Sequence alignment of ClcF against proteins of the non-redundant NCBI database indicated a high relatedness of the dehalogenase to the class of muconolactone delta-isomerases. Latter enzymes are found in the regular *ortho*-cleavage- or 3-oxoadipate pathway of catechol degradation and catalyze the conversion of (4*S*)-muconolactone into 3-oxoadipate enol-lactone (Fig. 1C). Like ClcF, all (methyl)muconolactone isomerases that have been investigated in that respect are able to dechlorinate (4*R*,5*S*)-5-chloromuconolactone to a mixture of *cis*- and *trans*-dienelactone [13,14]. However, this dechlorinating capability has only been shown *in vitro* for this class of enzymes and due to the restricted substrate range of catechol dioxygenases and muconate cycloisomerases does not play a physiological role. The same is true for the *in vitro* demonstration of muconolactone isomerase from *Cupriavidus necator* JMP134 to dechlorinate (4*R*,5*S*)- and (4*R*,5*R*)-5-chloro-3-methylmuconolactone, potential intermediates from 5-chloro-3-methylcatechol degradation [13,15,16]. Both reactions may be attributed as promiscuous activity towards these substrates. Thus, ClcF is likely to be an example of an evolutionary specialization of muconolactone isomerases for the promiscuous dechlorination activity. This hypothesis is strengthened by the observations that ClcF has almost completely lost the ability to convert (4*S*)-muconolatone to 3-oxoadipate enol-lactone [10] and, as already mentioned, that ClcF forms solely *cis*-dienelactone from 5CML-dechlorination whereas MLI-catalyzed dechlorinations result in mixtures of the *cis*- and *trans*-isomer of dienelactone.

Although a low-resolution structure of muconolactone isomerase of *Pseudomonas putida* is available for almost 30 years [17], the detailed enzymatic mechanism has not yet been elucidated. Based on reports of Stanier and Ornston [18] and Chari et al. [19], Pieper and co-workers [15] assumed that proton abstraction from C4 and proton addition to C2 would bring about the isomerization of muconolactone. They postulated that with 5-chloro-3-methylmuconolactone (and consequently also 5-chloromuconolactone) the proton abstraction from C4 must result in chloride elimination from C5.

In order to elucidate catalytically relevant residues, we report on the preparation and purification of mg amounts of recombinant ClcF as well as single amino acid exchange mutants of ClcF for the purpose of biochemical and X-ray crystallographic analysis. Mutants to be constructed were chosen based on the multiple sequence alignment with (methyl)muconolactone isomerases, on a low-resolution X-ray structure for muconolactone isomerase from *P. putida* [20] as well as on preliminary structural information for ClcF [21]. Kinetic studies of the wild-type protein and of these variants were to be performed in order to support crystallographic analysis and to interpret the catalytic function of certain amino acids. In addition, kinetic data were compared to those available for (methyl)muconolactone isomerases.

Materials and methods

Strains and plasmids and cultivation conditions

R. opacus 1CP was originally isolated from an enrichment culture with 2,4-dichlorophenol as the sole carbon source [22]. For DNA isolation, strain 1CP was grown at 30 °C in a mineral medium (pH 7.2) [23] with a doubled phosphate buffer concentration supplied with 20 g l^{-1} glycine and 2 g l^{-1} glucose. *P. putida* PRS2000 was grown at 30 °C in mineral medium (pH 7.2) [23] using 5 mM benzoate as the sole carbon source. *Escherichia coli* DH5α (GIBCO BRL) and *E. coli* BL21-CP(DE3)-RIL (Agilent Technologies, Stratagene) were grown aerobically with constant shaking at 37 °C in Luria-Bertani (LB) medium [24] containing 100 μg ml^{-1} ampicillin or a mixture of 100 μg ml^{-1} ampicillin and 35 μg ml^{-1} chloramphenicol, respectively.

Plasmids used in this study are summarized in Table S1 (Supplementary material).

Cloning of the clcF gene

Genomic DNA from *R. opacus* 1CP was obtained as described previously [10] and was used as the template in PCR amplification. The reaction mix for the PCR contained 0.5 μg template DNA, 5% (v/v) DMSO, 60 pmol of primer clcF_fw (GCTTCGA CATATGTTGTACCTAGTTC) and 60 pmol clcF_rev (ATGGATCCTCAGTCTTTGCCGAC) (Eurofins MWG-Biotech), 40 nmol dNTPs, 150 nmol Mg^{2+}, and 2.5 U Taq polymerase (Thermo Fisher Scientific, Fermentas) in a total volume of 100 μl. Both oligonucleotides contained recognition sites for the restriction endonucleases NdeI and BamHI, respectively (underlined sequence). The PCR product was initially ligated

into the T-tailed EcoRV-site of pBluescript II KS(+), yielding pBSclcF1a, and transformed into *E. coli* DH5α. The gene was then excised by digestion with NdeI and BamHI (Thermo Fisher Scientific, Fermentas) and ligated into the expression vector pET11a*. The resulting plasmid, pETRoclcF, was transformed into *E. coli* BL21-CP(DE)-RIL and used for expression purposes.

Site-directed mutagenesis to generate ClcF mutants

Mutants of ClcF were created according to the procedure described by Weiner et al. [25]. Briefly, primers carrying the desired mutation (Table S2, Supplementary material) were synthesized (biomers.net GmbH, Ulm, Germany) and the mutagenesis PCR with the wild-type plasmid as template was set up as described. Remaining parental plasmid DNA was removed by a digestion with DpnI and 1 μl of the mutagenic PCR was directly transformed into ultracompetent *E. coli* DH5α. Cells were plated onto LB-agar containing 100 μg ml^{-1} ampicillin. Several clones were picked and DNA was isolated (MinElute Plasmid Kit, Quiagen) and sequenced (Eurofins MWG-Biotech) in order to verify the incorporated mutation and the integrity of the gene sequence.

Expression of ClcF and its variants in E. coli BL21-CP-RIL

Due to the occurrence of codons AGG, AUA, and CUA which are rarely used by wild-type *E. coli*, strain BL21-CP(DE3)-RIL harbouring corresponding tRNA genes was chosen as an expression host for ClcF.

Freshly grown transformants of *E. coli* BL21-CP(DE3)-RIL harbouring the corresponding expression plasmid (e.g. pETRoclcF) were used to inoculate 10-ml LB-medium with 100 μg ml^{-1} ampicillin and 50 μg ml^{-1} chloramphenicol. After overnight cultivation at 30 °C under constant shaking (130 rpm), 5 ml of that pre-culture were used to inoculate 500 ml of the same medium to be incubated under similar growth conditions. At an optical density OD(600 nm) of 0.6–0.7, expression was induced by addition of 100 μM isopropyl-β-D-thiogalactopyranoside (IPTG). After incubation for further 4 h at 30 °C, the culture was harvested by centrifugation (5000×*g*, 15 min, 4 °C), washed with 54 mM Na/K-phosphate buffer (pH 7.2), and biomass was stored at −20 °C for further use.

Preparation of cell extracts

To freshly thawed biomass of expression clones, 0.5 U ml^{-1} Dnase I was added and disruption was achieved by two passages through a French pressure cell (1500 psi). The mixture was centrifuged (100,000×*g*, 45 min, 4 °C) and the clear supernatant served as cell-free crude extract for further protein purification. In cases, extracts were not immediately subjected to purification, the crude extract was stored at −20 °C.

Purification and storage of recombinant wild-type ClcF and its variants

For an initial heat treatment cell-free crude extract of the corresponding expression clone was incubated at 65 °C for 10 min. After rapid cooling to 4 °C on ice, precipitated protein was separated by centrifugation and removed (10,000×*g*, 15 min, 4 °C) resulting in a 3-fold increased specific activity of ClcF. The supernatant was then applied onto a Q-Sepharose column (1.6 cm × 4.0 cm, GE Healthcare) pre-equilibrated with 25 mM Tris–HCl, pH 7.5. The same buffer was used for the elution of unbound proteins which were shown to comprise also ClcF in concordance with its relatively high pI of 6.91. The wash eluate was then supplemented at 4 °C with solid ammonium sulfate to a final concentration of 1.6 M. After centrifugation (10,000×*g*, 15 min, 4 °C) the clear supernatant was loaded onto a Phenyl Sepharose column (2.6 cm × 2.8 cm, GE Healthcare) pre-equilibrated with high-salt buffer (25 mM Tris–HCl, pH 7.5, containing 1.6 M $(NH_4)_2SO_4$). ClcF and its variants were obtained as almost homogeneous preparations (purity ⩾97% according to SDS–PAGE) by means of a linear decreasing salt gradient (from 1.6 to 0 M ammonium sulfate). The enzyme eluted at ~1 M $(NH_4)_2SO_4$ and was precipitated by addition of solid ammonium sulfate to give a final concentration of 75% saturation. The suspension was incubated on ice for about 120 min and centrifuged (50,000×*g*, 15 min, 4 °C). Precipitated protein was stored either for short term at +4 °C or for longer times at −80 °C.

Determination of ClcF activity and protein concentration

ClcF activity was determined by measuring the formation of *cis*-dienelactone from (4*R*,5*S*)-5-chloromuconolactone at 280 nm in a Varian Cary 50 UV–Vis spectrophotometer using a specific extinction coefficient of 13.9 mM^{-1} cm^{-1} (this study). The same value was used for mutants E27D and N52A which yielded a mixture of *cis*- and *trans*-dienelactone. The standard assay consisted of 54 mM Na/K-phosphate buffer (pH 7.2), 100 μM (4*R*,5*S*)-5-chloromuconolactone [10], and was started by addition of the ClcF-containing sample.

Kinetic parameters *Km* and vmax were determined by means of the initial rate method assuming Michaelis–Menten kinetics. Reaction rates were fitted by non-linear least-square regression using the DynaFit 3.28 software [26]. The turnover number *kcat* was calculated with the molecular weight of 11,193.7 g mol^{-1} for one ClcF subunit. Initial reaction rates were determined in triplicates using the standard assay and substrate concentrations between 40 and 500 μM (4*R*,5*S*)-5-chloromuconolactone.

Protein concentrations were determined with the Bradford Protein Assay (BioRad) using bovine serum albumin as standard.

Product analysis of 5-chloromuconolactone turnover

ClcF variants were analyzed by reversed phase HPLC for their ability to dehalogenate 5-chloromuconolactone into *cis*- and *trans*-dienelactone. Twenty microliter samples from standard reaction assays (see below) were checked for complete transformation by UV/VIS-spectrophotometry and applied onto an Eurospher-100 C18 column (4 mm internal diameter, 125 mm length, Knauer, Berlin, Germany) as the stationary phase. An aqueous solution of 30% (v/v) methanol and 0.2% (w/v) H_3PO_4 served as the mobile phase at a flow rate of 0.7 ml min^{-1}. Absorption of the eluate was recorded at multiple wavelengths. Signals were assigned by comparison to external standards for *cis*-dienelactone (net retention volume 2.55 ml), *trans*-dienelactone (0.98 ml), (4*R*,5*S*)-5-chloromuconolactone (0.77 ml), and 2-chloromuconolactone (1.40 ml).

Determination of muconolactone-isomerizing activity

The ability of ClcF and its mutants to convert (4*S*)-muconolactone into 3-oxoadipate enol-lactone was assayed by a HPLC-based modification of the method of Ornston (1966) [27]. A typical reaction mixture contained in 1 ml: 25 μmol Tris–HCl (pH 7.5), 0.2 μmol (4*S*)-muconolactone, and approximately 0.01 U of partially purified 3-oxoadipate-enol-lactone hydrolase (ELH) from *P. putida* PRS2000 as an auxiliary enzyme. In order to determine the blank reaction rate, four 10-μl-samples were taken in the course of 90 min, quenched by addition of 5% (v/v) phosphoric acid and subjected to RP-HPLC (15% (v/v) MeOH, 2 g l^{-1} H_3PO_4, Eurospher-100 C18). Afterwards, 10 μl of 54 mM Na/K-phosphate buffer (pH 7.2), containing 5–10 mU (activity determined with 200 μM 5CML as the substrate) wild-type- or mutated ClcF were added to the assay mixture and RP-HPLC analysis was continued

for 90 min. The decrease of substrate (net retention volume 1.3 ml) was detected at 210 nm. A very slight decrease of (4*S*)-muconolactone, even in the absence of ClcF, indicated traces of muconolactone isomerase in the auxiliary enzyme preparation, however, this contamination was considered as a blank reaction rate.

The ELH was partially purified from a crude extract of benzoate-grown *P. putida* PRS2000 as described previously [10] using anion-exchange chromatography (Q-Sepharose, GE Healthcare) and hydrophobic interaction chromatography (Phenyl Sepharose, GE Healthcare). The final preparation of enriched 3-oxoadipate enol-lactone hydrolase was stored at −20 °C in 25 mM Tris–HCl (pH 7.5) in the presence of 50% (v/v) glycerol.

Purity control by SDS–PAGE

In order to determine the purity of wild-type ClcF and ClcF variants, enzyme preparations were subjected to discontinuous sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis. Slab gels with 15% acrylamide were prepared by using a Tris-/Tricine buffer system [28] and stained with Coomassie Brilliant Blue R-250.

Partial purification of muconolactone isomerase from P. putida PRS2000

Muconolactone isomerase (MLI) from *P. putida* was partially purified in order to measure and compare relative activities of this type of enzyme towards (4*S*)-muconolactone and 5CML. MLI was obtained during the purification process of enol-lactone hydrolase described before. During anion-exchange chromatography on Q-Sepharose, MLI eluted together with ELH at approximately 230 mM NaCl. Active fractions were pooled and after ammonium sulfate precipitation (75% saturation) proteins were subjected to hydrophobic interaction chromatography on Phenyl Sepharose with a decreasing gradient of 1.2–0 M $(NH_4)_2SO_4$ in 25 mM Tris–HCl (pH 7.5). MLI eluted at approximately 670–680 mM $(NH_4)_2SO_4$ followed by ELH (420–430 mM). Relative substrate specificities of partially purified MLI were tested with the standard assay for ClcF and the assay of the muconolactone-isomerizing activity, respectively, using 5CML and (4*S*)-muconolactone as substrates.

Sequence alignment and computational modelling

Conserved amino acids in (methyl)muconolactone isomerases and ClcF were determined by multiple sequence alignment using *ClustalW2* [29] (at http://www.ebi.ac.uk/Tools/msa/clustalw2/; default values). In order to visualize phylogenetic relatedness, a dendrogram was calculated by means of programs *Protdist* and *Fitch* of the PHYLIP package (version 3.69). Bootstrap values of which only those >50% are shown were derived from *Seqboot* (PHYLIP) calculations with 100 replicates.

The tertiary structure modelling of ClcF was conducted applying the muconolactone isomerase from *P. putida* (PDB_ID:1MLI) as template [20]. The protein fold recognition server PHYRE2 [30] was used in an automated procedure and images were generated with *Pymol* [31]. Structural manipulations were made using the crystallographic object-oriented tool kit *Coot* [32].

Chemicals

(4*R*,5*S*)-5-Chloromuconolactone, 2-chloromuconolactone, and (4*S*)-muconolactone were enzymatically prepared from 2-chloro-*cis,cis*-muconate and *cis,cis*-muconate [33], respectively, using purified muconate cycloisomerase from *P. putida* PRS2000 [12]. *cis*-Dienelactone and *trans*-dienelactone were available by chemical synthesis [34].

Results and discussion

Construction of a ClcF expression clone and preparation of recombinant 5-chloromuconolactone dehalogenase

In order to obtain sufficient quantities of ClcF to investigate its kinetic parameters and for future structure determination, the corresponding ORF was cloned into the cloning vector pBluescript II KS(+). Amplification by two *clcF*-specific primers clcF_fw and clcF_rev yielded an amplicon of expected size (303 bp) whose identity was confirmed by DNA sequence analysis. The gene *clcF* was subsequently ligated into the expression vector pET11a* and designated as pETRoclcF. Expression of the wild-type *clcF* gene in *E. coli* BL21-CP(DE3)-RIL at 30 °C in the presence of 100 μM IPTG led to almost exclusive formation of the protein in soluble active form. Thus, the chosen expression strain with t-RNAs for codons not usually occurring in *E. coli* was obviously suitable for expression of *R. opacus clcF*.

Relatedness of ClcF to (methyl)muconolactone isomerases and identification of conserved residues for site-directed mutagenesis

In order to identify further representatives of ClcF-like proteins, a BLASTP search was made against available protein sequences of the non-redundant database of the NCBI. ClcF was found to match the superfamily of muconolactone delta-isomerase (EC 5.3.3.4) [27] since a large number of (putative) representatives of this enzyme family showed high similarities (46–61% identity) over a query coverage of 95–100% (Fig. 2A). Analyses of the relatedness between ClcF and some representatives of these matches showed that the dehalogenase branches deep within the dendrogram of regular (methyl)muconolactone isomerases (Fig. 2B). That all hypothetical muconolactone isomerases used in the dendrogram indeed belong to the regular EC-5.3.3.4-type of isomerase was assured by considering only those representatives genes of which were part of a complete catechol-catabolic operon. None of the obtained protein hits with similarity to ClcF was found to be encoded in close neighborhood to genes of chlorocatechol degradation. This finding and the fact that the similarity of the best hit is restricted to 61% identical positions makes a physiological function in dehalogenation at least unlikely. Thus, ClcF of *R. opacus* 1CP is suggested to be the only available representative of a 5CML dehalogenase, so far.

Homology of ClcF and muconolactone isomerase is substantiated by comparison of the tertiary and quaternary protein structures of both enzymes. MLI of *P. putida* has been structurally resolved at 7 Å [17] and later on at 3.3 Å [20] and was found to exhibit a decameric structure with 5-fold and 2-fold symmetries. Each subunit is composed of a body with two alpha-helices, an anti-parallel twisted beta-sheet of four strands, and an extended arm. The helices and the sheet form a two-layered structure with an enclosed hydrophobic core and a partially formed putative active-site pocket. The latter one is completed by means of the C-terminal arm of another subunit.

A basically similar structure was found for ClcF [21] and calculation of the alpha-C-backbone of the dehalogenase allowed to identify those amino acids which are closely located to the putative active site and thus eventually are involved in substrate binding and catalysis (Fig. 3). Residues Glu27, Tyr50, Asn52, and His87 are of particular interest, since they are highly conserved in MLIs and are shared by ClcF. Another interesting residue is Ser89. The observation that this residue is highly conserved in MLIs but

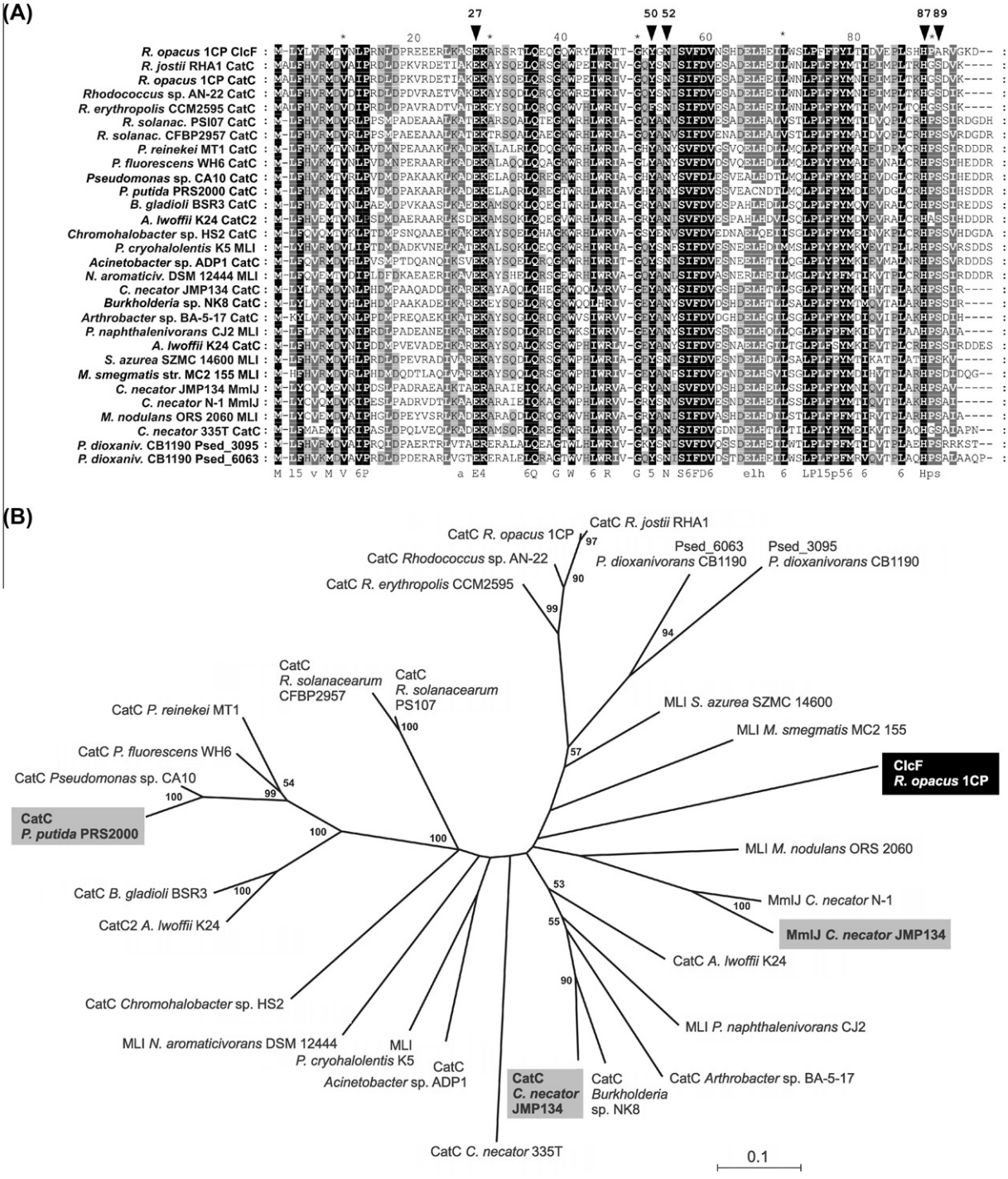

Fig. 2. (A) Identification of conserved amino acids in (putative) (methyl)muconolactone isomerases and the novel 5-chloromuconolactone dehalogenase ClcF and (B) dendrogram showing the relatedness between selected representatives of these proteins. Highly conserved amino acids glutamic acid (pos. 27), tyrosine (pos. 50), asparagine (pos. 52), histidine (pos. 87), and serine (pos. 89), which were subjected to modification in the present study are marked. Protein accession numbers are as follows: *Acinetobacter lwoffii* K24, CatC, AAC46227; *A. lwoffii* K24, CatC2, AAC31768; *Acinetobacter* sp. ADP1, AAC46431; *Arthrobacter* sp. BA-5-17, BAD11153; *Burkholderia gladioli* BSR3, AEA64672; *Burkholderia* sp. NK8, BAB21459; *C. necator* 335T, AAG42025; *C. necator* JMP134, CatC, AAZ61055; *C. necator* JMP134, MmlJ, CAA67959; *C. necator* N-1, AEI81695; *Chromohalobacter* sp. HS2, ABV82779; *Methylobacterium nodulans* ORS 2060, ACL57349; *Mycobacterium smegmatis* MC2 155, ABK73752; *Novosphingobium aromaticivorans* DSM 12444, ABP64688; *Polaromonas naphthalenivorans* CJ2, ABM37427; *Pseudomonas fluorescens* WH6, EEY97020; *P. putida* PRS2000, AAA25767; *Pseudomonas reinekei* MT1, ABI93946; *Pseudomonas* sp. CA10, BAB32457; *Pseudonocardia dioxanivorans* CB1190, MLI Psed_3095, AEA25292; *P. dioxanivorans* CB1190, MLI Psed_6063, AEA28172; *Psychrobacter cryohalolentis* K5, ABE75050; *Ralstonia solanacearum* CFBP2957, CBJ43151; *R. solanacearum* PSI07, CBJ51252; *Rhodococcus erythropolis* CCM2595, CAX36484; *Rhodococcus jostii* RHA1, ABG94176; *Rhodococcus opacus* 1CP: ClcF, CAD28145; CatC, CAA67935; *Rhodococcus* sp. AN-22, BAE46389; *Saccharomonospora azurea* SZMC 14600, EHK84170.

exchanged by alanine in ClcF might be of relevance for the almost complete inability of ClcF to convert muconolactone or for the high product specificity of ClcF to yield pure *cis*-dienelactone from 5CML-turnover. Glu27, His87, and Ser89 have already been proposed by Katti and coworkers [20] to be of catalytic relevance in muconolactone isomerase.

A simple acid-base-catalyzed 1,3-proton shift was identified as the mechanism for muconolactone isomerization, in which

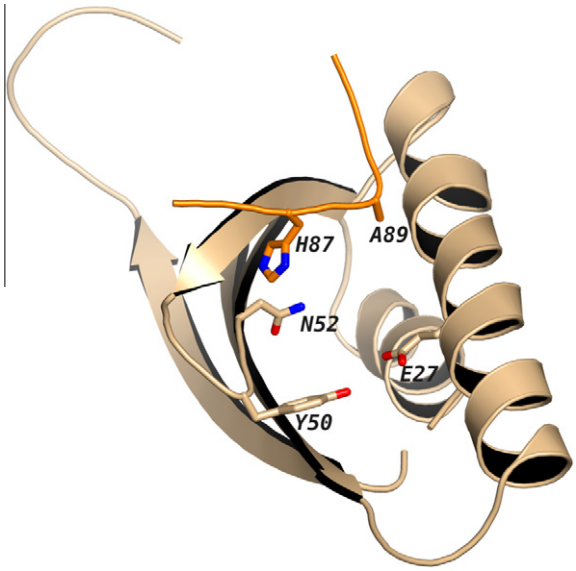

Fig. 3. Homology model of ClcF showing the location of mutated residues around the putative active-site pocket. One subunit was expanded to asymmetry using a fragment of a second neighboured monomer to model the position of the C-terminal residues H87 and A89 in the active site.

deprotonation at C-4 is followed by a shift of the double bond and a final protonation of C-2 [15,19] (Fig. 4A). Despite the fact that a similar mechanism has been reported for ketosteroid isomerase [35], sequence alignment and structural comparison of this type of enzyme with ClcF and MLIs do not indicate relatedness (data not shown). Early attempts to construe the peptide (Tyr-Gly-Asn-Ile-Ser) of MLI of *P. putida* to be homologous to an active-site tetrapeptide (Tyr-Ala-Asn-Ser) of ketosteroid isomerase [20,36] could not be confirmed in the light of advanced structural studies [37].

In order to gain evidence for an involvement of the above mentioned amino acid residues in substrate binding and catalytic mechanism, ClcF mutants E27Q, E27D, Y50A, N52A, H87A, and A89S were created. Except mutant H87A all variants could be expressed as soluble proteins by means of a pET11a*/*E. coli* BL21-CP(DE3)-RIL expression system.

Purification of recombinant ClcF and its variants

ClcF and variants thereof were expressed recombinantly in *E. coli* and purified by a procedure similar to the one used for the enrichment of the wild-type enzyme from *R. opacus* 1CP [11]. The combination of a heat treatment (65 °C), an anion-exchange- and a hydrophobic interaction chromatography resulted in a preparation that contained 37% of the initial ClcF activity. SDS–PAGE of the preparation yielded a single band of approx. 11 kDa (theoretical MW 11,193.72 Da) and indicated homogeneity sufficient for crystallisation approaches ($\geqslant$97%, data not shown).

The purification of all ClcF variants was achieved by basically the same procedure. While mutants E27Q, E27D, Y50A, N52A, and A89S were obtained as soluble proteins, expression of variant H87A yielded solely inclusion bodies.

Kinetics of 5CML-conversion by (methyl)muconolactone isomerases from C. necator JMP 134 and by ClcF and its variants

Comparison of kinetic parameters of ClcF for 5CML-conversion to available data of (methyl)muconolactone isomerases should indicate an evolutionary specialization of the unique 5-chloromuconolactone dehalogenase for the process of dechlorination. In fact, ClcF was shown to exhibit the highest specificity ($1.4 \times 10^6\ M^{-1}\ s^{-1}$) towards (4*R*,5*S*)-5-chloromuconolactone when compared to MLI ($5.8 \times 10^5\ M^{-1}\ s^{-1}$) as well as of MMLI ($6.1 \times 10^4\ M^{-1}\ s^{-1}$) from *C. necator* JMP134 (Table 1). The extremely high maximum turnover number of 5CML by MLI ($5950 \pm 770\ s^{-1}$) which exceeds that of ClcF ($529 \pm 26\ s^{-1}$) by a factor of 11.2 is compensated by its lower substrate affinity as it is expressed by a 27.4-fold higher Michaelis constant of MLI (10,300 ± 1800 μM).

The kinetic parameters of all ClcF variants for 5CML-conversion were found to be significantly changed. Compared to the five mutants E27D, E27Q, Y50A, N52A, and A89S, wild-type ClcF shows the highest *kcat* value of $529 \pm 26\ s^{-1}$. However, the mutant A89S turned out to be slightly more specific for (4*R*,5*S*)-5-chloromuconolactone conversion compared to the wild-type enzyme. The slight increase of the specificity constant of the A89S variant by a factor of 1.2 results mainly from a significantly reduced *Km* value, which is compensated in part by the reduced turnover number.

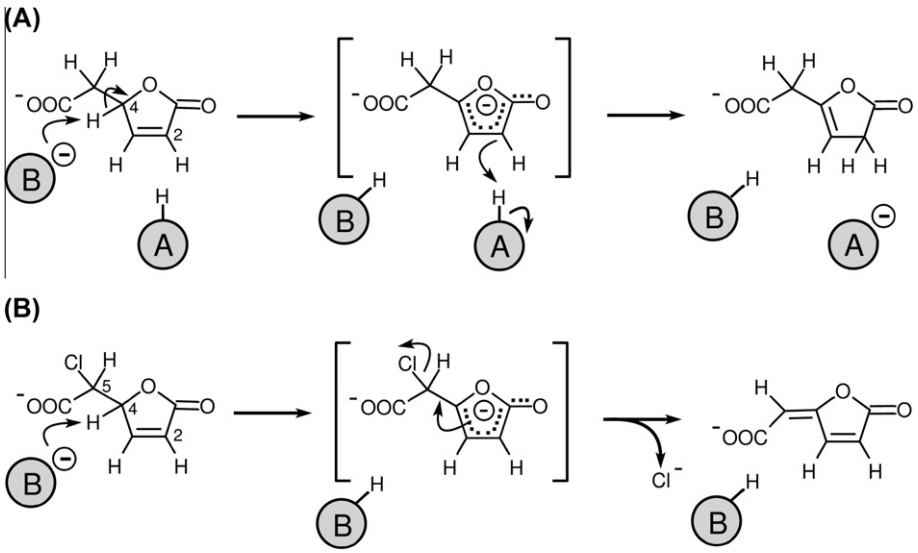

Fig. 4. (A) Proposed mechanism of muconolactone isomerase catalysis [20,38] and (B) hypothetical adaptation of the initial proton abstraction in the dehalogenation of 5-chloromuconolactone. The 1,3-proton transfer in muconolactone involves an initial proton abstraction at C-4 by a basic amino acid residue, followed by protonation of the carbanion intermediate at C-2 through the action of an acidic amino acid residue [38]. During 5CML-conversion the negative charge of carbanion transition state is removed by chloride elimination. Pointed lines indicate negatively charged resonance structures.

Table 1
Comparison of kinetic parameters for the conversion of (4*R*,5*S*)-5-chloromuconolactone by wild-type ClcF, some variants, and (methyl)muconolactone isomerases from *C. necator* JMP134.

Enzyme	*Km* [μM]	vmax [U mg^{-1}]	*kcat* [s^{-1}][b]	*kcat/Km* [M^{-1} s^{-1}]
ClcF wild-type	376 ± 32	2838 ± 141	529 ± 26	1.4×10^6
ClcF (E27D)	303 ± 10	11.5 ± 0.2	2.10 ± 0.04	6.9×10^3
ClcF (E27Q)	307 ± 12	0.45 ± 0.01	0.080 ± 0.002	261
ClcF (Y50A)	1556 ± 127	137 ± 9	25.6 ± 1.8	1.6×10^4
ClcF (N52A)	1250 ± 112	26.8 ± 2.0	5.0 ± 0.4	4.0×10^3
ClcF (H87A)	N. d. (inclusion bodies)			
ClcF (A89S)	215 ± 25	1934 ± 109	361 ± 20	1.7×10^6
MLI JMP134[a]	10,300 ± 1800	35,300 ± 4600	5950 ± 770	5.8×10^5
MMLI JMP134[a]	5200 ± 800	1850 ± 200	317 ± 35	6.1×10^4

N. d. not determined.
[a] [14,15].
[b] *kcat*-values were calculated on the basis of a subunit size of 11.2 kDa (ClcF and variants), 10.1 kDa (muconolactone isomerase, MLI), and 10.3 kDa (methylmuconolactone isomerase, MMLI).

Considering that serine is highly conserved among all available (methyl)muconolactone isomerases the fact that alanine instead of serine at this position does positively affect the performance of ClcF to convert 5CML raises the question about evolutionary reasons for this exchange. Possible explanations may be related to the inability of ClcF to convert (4*S*)-muconolactone or to the product specificity of dechlorination (*cis*- and *trans*-isomer of dienelactone).

A drastic effect on activity was observed for the substitution of the highly conserved E27 against aspartic acid or glutamine leading to a decrease of the turnover number by a factor of around 250 (E27D) or 6600 (E27Q), respectively. Obviously the acidic residue at position 27 plays a crucial role in the turnover of (4*R*,5*S*)-5-chloromuconolactone. The substrate affinities were not significantly affected by both mutations, a fact which suggests that E27 may not primarily be involved in substrate binding. With a p*Ka* value of 4.2 the carboxyl group should be almost completely ionized under physiological conditions (pH 7.0) and thus act primarily as a base. For the mechanism of muconolactone isomerization a simple acid-base catalysis *via* a proton transfer from C-4 to C-2 has been suggested (Fig. 4A) [19,20] and it is very likely that an initial step of proton abstraction is also involved in the dechlorination of 5CML (Fig. 4B). Despite of the presence of a β-carboxyl function with similar acidity (p*Ka* = 3.9) in the aspartic acid residue of mutant E27D, the catalytic activity is drastically reduced when compared to the enzyme with glutamic acid. This presumably is a result of an insufficient length of the aspartate side chain impeding contact to the substrate (4*R*,5*S*)-5-chloromuconolactone.

The exchanges of Y50 and N52 for alanine were accompanied with decreases of activity by factors 21 and 106, respectively. In addition to the strong effects on enzyme activity, the effects of both exchanges on *Km* were significant with increases of around three (N52A) to four (Y50A). Thus, N52 and Y50 may be involved in substrate fixation *via* hydrogen bonding interactions.

Influence of amino acid exchanges on (4S)-muconolactone conversion

Initial attempts to characterize the substrate specificity of ClcF pointed to a complete inability to transform (4*S*)-muconolactone [10]. The availability of larger amounts of the recombinant enzyme now allowed the detection of a very low specific activity (1.9 U mg^{-1}) which corresponds to 0.2% of the activity determined with (4*R*,5*S*)-5-chloromuconolactone (988 U mg^{-1}) at identical substrate concentration (200 μM) (Table 2). In contrast, muconolactone isomerase of *P. putida* PRS2000 converts (4*S*)-muconolactone compared to 5CML with a relative activity of 170% under similar conditions. A similar capability to transform both (4*R*,5*S*)-5-chloromuconolactone and (4*S*)-muconolactone has also been reported for muconolactone isomerase and methylmuconolactone isomerase from *C. necator* JMP134 [13,15].

In order to identify single residues which are responsible for the increase of substrate specificity of ClcF, mutants were characterized for their ability to convert (4*S*)-muconolactone. Unlike the dehydrochlorination of 5-chloromuconolactone to *cis*-dienelactone, which is an irreversible reaction, the isomerization of (4*S*)-muconolactone to 3-oxoadipate enol-lactone has a pronounced equilibrium character making it necessary to determine kinetics in the presence of an excess of 3-oxoadipate enol-lactone hydrolase [27]. Specific activities of wild-type ClcF and variants towards (4*S*)-muconolactone were determined in the presence of 200 μM of this substrate. Compared to the wild-type enzyme (1.9 U mg^{-1}) only mutant A89S showed an increased specific activity of 3.0 U mg^{-1} for the non-chlorinated parent compound whereas all other mutants were shown to fall below these values for two to six orders of magnitude (Table 2). A quotient of specific activity towards ML and 5CML was calculated and normalized for wild-type ClcF in order to better visualize changes in substrate preference. Mutants E27D and N52A showed a relative increase of activity for ML, however, due to the strong decrease of overall activity is practically irrelevant when been compared to the corresponding activity of the wild-type. Only for mutant A89S, of which the specificity constant of 1.7×10^6 M^{-1} s^{-1} exceeds that of wild-type ClcF by a factor of 1.2 (Table 1), an 1.6-fold increase of muconolactone-isomerizing activity is accompanied with an absolute enhancement of specific activity under the conditions of measurement. Interestingly, this serine residue was shown to be highly conserved among all muconolactone isomerases and only in ClcF is exchanged by alanine.

Effect of amino acid exchanges on product formation from 5CML

As mentioned before, isomerization of (4*S*)-muconolactone comprises a deprotonation at C-4 and, in order to compensate the resulting negative charge of the carbanion, a subsequent protonation of C-2 (Fig. 4A). Only the first of both steps should be necessary for the conversion of 5CML, since here it is the release of chloride which removes the negative charge of the transition state (Fig. 4B). For 5CML-conversion the second step is supposed to be a spontaneous process which in principal does not need the presence of or an interaction with a certain amino acid residue, a hypothesis which is strengthened by the observation that slightly alkaline conditions (pH 9) are sufficient to cause an immediate dehydrohalogenation of 5CML in the absence of an enzyme [12].

However, comparison of chemical and enzymatic 5CML-conversion indicates a distinctive influence of protein structure on product configuration. While alkaline treatment of 5CML yields

Table 2
Conversion of (4*S*)-muconolactone by wild-type ClcF, some variants, and muconolactone isomerase from *P. putida* PRS2000, and comparison of product specificity from (4*R*,5*S*)-5-chloromuconolactone turnover.

Enzyme	Sp. activity for ML[a] [U mg^{-1}]	Sp. activity for 5CML[a] [U mg^{-1}]	Normalized ratio (sp. act. for 5CML)/(sp. act. for ML)[b]	Product(s) from 5CML-conversion *cis*-DL [%]/*trans*-DL [%]
ClcF wild-type	1.9	975	1	100/0
ClcF (E27D)	0.023	2.81	4.1	100/0
ClcF (E27Q)	3.7×10^{-6}	0.177	0.01	92/8
ClcF (Y50A)	6.9×10^{-3}	15.7	0.22	100/0
ClcF (N52A)	0.025	3.76	3.3	75/25
ClcF (A89S)	3.0	926	1.6	100/0
MLI *P. putida* PRS2000	N. d.	N. d.	172[c]	65/35

N. d. not determined.
[a] Substrate concentration = 200 μM.
[b] The ratio (sp. act. for 5CML)/(sp. act. for ML) of a mutant was calculated and referred to that of ClcF wild-type (normalized as 1).
[c] Determined from partially purified enzyme on the basis of volume activities.

predominantly the *trans* isomer of dienelactone ((2*Z*)-2-(5-oxo-2(5*H*)-furanylidene)acetic acid) (~87% *trans*- and ~13% *cis*-dienelactone [this study]; >95% *trans*-dienelactone [13]) via *anti*-elimination, enzymatic conversions favour formation of the *cis*-isomer (=(2*E*)-2-(5-oxo-2(5*H*)-furanylidene)acetic acid) via *syn*-elimination. MLI of *P. putida* PRS2000 was shown in this study to convert 5CML into 65% *cis*- and 35% *trans*-dienelactone (Table 2), a preference similar to the ones reported for MLI and MMLI from *C. necator* JMP134 yielding mixtures of *cis*- and *trans*-dienelactone of 3:1 and 2.5:1, respectively [14,15]. A remarkable difference in product specificity was found for ClcF of *R. opacus* 1CP, which yields almost pure *cis*-dienelactone. Since the product configuration does reflect the localization of the carboxylic group in the moment of chloride elimination, product specificity is likely to be influenced by the extent of rotatability of the exocyclic C4–C5 single bond.

During the conversion of muconolactone by MLI, a free rotatability of this single bond does not have an effect on product formation, since this structural moiety is not affected during the course of reaction. Hence, a fixation of the carboxylic acid residue should be necessary for the purpose of substrate binding rather than for the reaction mechanism itself.

The remarkably high product specificity of ClcF makes sense from a physiological point of view, since the activity of the following 3-chlorocatechol-catabolic enzyme dienelactone hydrolase (ClcD2) is restricted to *cis*-dienelactone [10]. Recruitment of a regular MLI for the purpose of dechlorination might lead to the formation of *trans*-dienelactone as a dead-end metabolite in strain 1CP. Thus, a high product specificity of ClcF could be easily interpreted as an evolutionary adaptation of MLI in order to meet the narrow substrate requirements of the following catabolic enzyme.

Available variants were tested for an effect of the mutated single amino acid residue on product specificity.

While mutants E27D, Y50A, and A89S behaved like wild-type ClcF and did not produce measurable amounts of *trans*-dienelactone, variants E27Q and N52A led to the formation of 8% and 25%, respectively, of the *trans* isomer. The behavior of the latter variant can be interpreted, if it is assumed that asparagine is involved in substrate binding of the carboxyl group by hydrogen bonds. However, since N52 is conserved in all MLIs other structural features than this residue are responsible for the high specificity of ClcF.

Conclusions

The ClcF protein of *R. opacus* 1CP could successfully be expressed and purified from *E. coli* BL21-CP-RIL(pETRoclcF) in mg-amounts and to homogeneity, sufficient for further structural studies. A three-step procedure with heat treatment, Q-Sepharose- and Phenyl Sepharose chromatography turned out to be a reliable purification method for ClcF and single-position mutants. All variants investigated in this study showed significant to drastic effects either on substrate binding (Y50A, N52A), on the reaction rate of catalysis (E27D, E27Q) or on both processes (A89S). In addition, N52 was found to strongly affect product specificity of dehydrochlorination probably by the hindrance of rotatability of the exocyclic C–C single bond of the carbanion transition state prior to elimination. This fixation could be achieved by hydrogen bonds between arginine and the carboxyl group of 5CML. None of the investigated variants was able to fully regain the evolutionarily lost ability of ClcF to convert (4*S*)-muconolactone and the relative activity towards this compound could only be increased by a few percent for variants E27D and N52A. In combination with crystallographic data of ClcF which are currently in progress the obtained data will help to interpret the exact role of residues in the catalytic process.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.abb.2012.07.007.

References

[1] W. Reineke, C. Mandt, S.R. Kaschabek, D.H. Pieper, Chlorinated Hydrocarbon Metabolism, eLS, John Wiley & Sons Ltd., Chichester, 2011. <http://www.els.net> http://dx.doi.org/10.1002/9780470015902.a0000472.pub3.
[2] T.D. DiStefano, J.M. Gossett, S.H. Zinder, Appl. Environ. Microbiol. 57 (1991) 2287–2292.
[3] P.J.M. Middeldorp, M.L.G.C. Luiyten, B.A. van de Pas, M.H.A. van Eekert, S.W.M. Kengen, G. Schraa, A.J.M. Stams, Biorem. J. 3 (1999) 151–169.
[4] I. Bartels, H.J. Knackmuss, W. Reineke, Appl. Environ. Microbiol. 47 (1984) 500–505.
[5] C.A. Pettigrew, B.E. Haigler, J.C. Spain, Appl. Environ. Microbiol. 57 (1991) 157–162.
[6] W. Reineke, H.J. Knackmuss, Annu. Rev. Microbiol. 42 (1988) 263–287.
[7] M. Schlömann, Biodegradation 5 (1994) 301–321.
[8] I.P. Solyanikova, O.V. Maltseva, M.D. Vollmer, L.A. Golovleva, M. Schlömann, J. Bacteriol. 177 (1995) 2821–2826.
[9] D. Eulberg, E.M. Kourbatova, L.A. Golovleva, M. Schlömann, J. Bacteriol. 180 (1998) 1082–1094.
[10] O.V. Moiseeva, I.P. Solyanikova, S.R. Kaschabek, J. Gröning, M. Thiel, L.A. Golovleva, M. Schlömann, J. Bacteriol. 184 (2002) 5282–5292.
[11] O.V. Moiseeva, O.V. Belova, I.P. Solyanikova, M. Schlömann, L.A. Golovleva, Biokhimiya 66 (2001) 678–687.
[12] M.D. Vollmer, P. Fischer, H.J. Knackmuss, M. Schlömann, J. Bacteriol. 176 (1994) 4366–4375.
[13] M. Prucha, V. Wray, D.H. Pieper, Eur. J. Biochem. 237 (1996) 357–366.
[14] M. Prucha, A. Peterseim, D.H. Pieper, Arch. Microbiol. 168 (1997) 33–38.

[15] M. Prucha, A. Peterseim, K.N. Timmis, D.H. Pieper, Eur. J. Biochem. 237 (1996) 350–356.
[16] K. Pollmann, V. Wray, D.H. Pieper, J. Bacteriol. 187 (2005) 2332–2340.
[17] B.A. Katz, D. Ollis, H.W. Wyckoff, J. Mol. Biol. 184 (1985) 311–317.
[18] R.Y. Stanier, L.N. Ornston, Adv. Microb. Physiol. 9 (1973) 89–151.
[19] R.V.J. Chari, C.P. Whitman, J.W. Kozarich, K.-L. Ngai, L.N. Ornston, J. Am. Chem. Soc. 109 (1987) 5520–5521.
[20] S.K. Katti, B.A. Katz, H.W. Wyckoff, J. Mol. Biol. 205 (1989) 557–571.
[21] C. Roth, S.R. Kaschabek, J.A.D. Gröning, T. Handrek, M. Schlömann, N. Sträter, Sect. F: Struct. Biol. Cryst. Commun. F68 (2012) 591–595.
[22] S.N. Gorlatov, O.V. Maltseva, V.I. Shevchenko, L.A. Golovleva, Mikrobiologiya 58 (1989) 802–806.
[23] E. Dorn, M. Hellwig, W. Reineke, H.J. Knackmuss, Arch. Microbiol. 99 (1974) 61–70.
[24] J. Sambrook, E.F. Fritsch, T. Maniatis, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Plainview, New York, 1989.
[25] M.P. Weiner, G.L. Costa, W. Schoettlin, J. Cline, E. Mathur, J.C. Bauer, Gene 151 (1994) 119–123.
[26] P. Kuzmic, Anal. Biochem. 237 (1996) 260–273.
[27] L.N. Ornston, J. Biol. Chem. 241 (1966) 3795–3799.
[28] H. Schägger, G. von Jagow, Anal. Biochem. 166 (1987) 368–379.
[29] M. Goujon, H. McWilliam, W. Li, F. Valentin, S. Squizzato, J. Paern, R. Lopez, Nucleic Acids Res. 38 (2010) W695–W699.
[30] L.A. Kelley, M.J.E. Sternberg, Nat. Protocols 4 (2009) 363–371.
[31] W.L. DeLano, The PyMOL Molecular Graphics System, DeLano Scientific, Palo Alto, CA, USA, 2002.
[32] P. Emsley, B. Lohkamp, W.G. Scott, K. Cowtand, Acta Crystallogr. D. Biol. Crystallogr. 66 (2010) 486–501.
[33] S.R. Kaschabek, W. Reineke, J. Org. Chem. 59 (1994) 4001–4003.
[34] R.A. Massy-Westropp, M.F. Price, Aust. J. Chem. 33 (1980) 333–341.
[35] S.K. Malhotra, H.J. Ringold, J. Am. Chem. Soc. 87 (1965) 3228–3236.
[36] T.M. Penning, P. Talalay, J. Biol. Chem. 256 (1981) 6851–6858.
[37] N.-C. Ha, G. Choi, K.Y. Choi, B.-H. Oh, Curr. Opin. Struct. Biol. 11 (2001) 674–678.
[38] K.-L. Ngai, Enzymes of the beta-ketoadipate pathway in *Pseudomonas putida*, PhD-thesis (1981) University of Pennsylvania.