

Identification, Cloning, and Characterization of a Novel Ketoreductase from the Cyanobacterium *Synechococcus* sp. Strain PCC 7942[†]

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A new ketoreductase useful for asymmetric synthesis of chiral alcohols was identified in the cyanobacterium *Synechococcus* sp. strain PCC 7942. Mass spectrometry of trypsin-digested peptides identified the protein as 3-ketoacyl-[acyl-carrier-protein] reductase (KR) (EC 1.1.1.100). The gene, referred to as *fabG*, was cloned, functionally expressed in *Escherichia coli*, and subsequently purified to homogeneity. The enzyme displayed a temperature optimum at 44°C and a broad pH optimum between pH 7 and pH 9. The NADPH-dependent KR was able to asymmetrically reduce a variety of prochiral ketones with good to excellent enantioselectivities (>99.8%). The KR showed particular high specific activity for asymmetric reduction of ethyl 4-chloroacetoacetate (38.29 ± 2.15 U mg⁻¹) and 2',3',4',5',6'-pentafluoroacetophenone (8.57 ± 0.49 U mg⁻¹) to the corresponding (*S*)-alcohols. In comparison with an established industrial enzyme like the alcohol dehydrogenase from *Lactobacillus brevis*, the KR showed seven-times-higher activity toward 2',3',4',5',6'-pentafluoroacetophenone, with a remarkably higher enantiomeric excess (>99.8% [*S*] versus 43.3% [*S*]).

Chiral alcohols represent important intermediates in the synthesis of optically active compounds. One of the most efficient ways to produce chiral alcohols of high purity is by biocatalytical reduction of ketones. Although the use of whole cells and enzymes in industrial processes is expected to increase, the practicability of technical applications is often limited by the lack of suitable biocatalysts (25). Despite considerable progress in the field of protein engineering, there is intense interest in finding new enzymes with desired properties.

Phototrophic microorganisms have enzymes meeting diverse demands from the production of chiral alcohols. Different cyanobacteria have been shown to reduce halogenated acetophenones to the corresponding alcohols with high stereoselectivity (7, 13). In particular, fluorinated building blocks play an increasing part as precursor compounds for small-molecule drugs, e.g., for the antidepressant bupropion (21) or for celecoxib (15), which is used for treatment of rheumatoid arthritis. Widespread enzymes that catalyze asymmetric synthesis, e.g., alcohol dehydrogenases from *Lactobacillus* species, or enantioselective hydrolysis, e.g., lipases from *Pseudomonas* species, exhibited low stereoselectivities for fluorinated compounds (7, 22). In contrast, different cyanobacteria reached excellent enantioselectivities with the perfluorinated molecule 2',3',4',5',6'-pentafluoroacetophenone (7, 13). However, large-scale cyanobacterial biotransformations are technically not feasible due to the enormous photobioreactors required. Although cyanobacteria were shown to perform asymmetric reductions of prochiral ketones completely uncoupled from photosynthesis (8), the addition of light is still necessary for the generation of biomass.

For these reasons, enzymes from cyanobacteria represent interesting targets for use in industrial processes by heterologous expression. In this article, we describe the identification and characterization of a novel ketoreductase from the unicellular cyanobacterium *Synechococcus* sp. strain PCC 7942, which catalyzed, among others, the reduction of the hard-to-convert ketone 2',3',4',5',6'-pentafluoroacetophenone.

MATERIALS AND METHODS

Chemicals and enzymes. The prochiral ketones and chiral (*R*)- or (*S*)-alcohols under investigation were purchased from Merck (Darmstadt, Germany), Sigma-Aldrich (Schnellendorf, Germany), Alfa Aesar (Karlsruhe, Germany), Jülich Chiral Solutions (Jülich, Germany), or Apollo Scientific (Stockport, United Kingdom) with purum grade. Enzymes were obtained from New England Biolabs (Frankfurt, Germany) unless otherwise indicated. All amplifications used cloned *Pfu* polymerase from Stratagene (Amsterdam, The Netherlands). DNA samples were purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA) according to the manufacturer's protocols. Primers were purchased from MWG Biotech (Ebersberg, Germany).

Bacterial strains and plasmids. The cyanobacterial strain *Synechococcus* sp. strain PCC 7942 was provided by the Pasteur Culture Collection of cyanobacteria (Paris, France). *Escherichia coli* strains XL-10 Gold (Stratagene, Amsterdam, The Netherlands) and Tuner (DE3) (Novagen, Madison, WI) were used for cloning and overexpression experiments, respectively. The plasmid pETM-41 (EMBL, Heidelberg, Germany) was used as a cloning and expression vector containing a hexahistidine-maltose-binding-protein (His₆-MBP) tag followed by a tobacco etch virus (TEV) protease cleavage site. The plasmid pRK793 (11), used for expression of a His₆-tagged TEV protease, was obtained in a bacterial culture of *E. coli* BL21(DE3) CodonPlus RIL cells from the Addgene Plasmid Repository (Cambridge, MA). The plasmid pBtac-*adh*_{L.brevis} was a kind gift from S. Bringer-Meyer (Research Centre Jülich, Jülich, Germany). *E. coli* BL21(DE3)(pBtac-*adh*_{L.brevis}) cells were constructed as described previously (4).

Protein purification from *Synechococcus* sp. strain PCC 7942. *Synechococcus* cells were cultivated on a 20-liter scale in BG-11 medium (1) according to the method of Franco-Lara et al. (6). For cell disruption, 95 mg ml⁻¹ of *Synechococcus* sp. strain PCC 7942 was suspended in 50 mM Tris-HCl (pH 8)–1 mM EDTA–0.1 mM phenylmethylsulfonyl fluoride (PMSF)–0.5 g liter⁻¹ lysozyme and incubated at 30°C for 2 h. After ultrasonication for 40 s on ice, the membranes were separated by sedimentation at 17,000 × *g* at 4°C for 10 min. The soluble protein fraction was freeze-dried for later purification. Lyophilized protein (0.1 g [dry weight] liter⁻¹) was solved in water and separated from small cell debris by sedimentation at 100,000 × *g* for 1 h at 4°C with an SW 40 Ti rotor

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(Beckmann Coulter, Krefeld, Germany). The soluble protein fraction was separated by size exclusion chromatography (SEC) using a HiLoad 26/60 Superdex 200 column (Amersham, Uppsala, Sweden) at flow rates of 2 ml min⁻¹ (20 mM Tris-HCl buffer [pH 8], 200 mM NaCl, 1 mM EDTA). Proteins were detected by absorbance at 206 nm. After dialysis (molecular weight cutoff, 14 kDa) against 50 mM Tris-HCl (pH 8)–1 mM EDTA–0.1 mM PMSF, the resulting sample was loaded onto a 60-ml column of DEAE52 anion exchange resin (Serva, Heidelberg, Germany) and eluted using a gradient from 0 to 3 M NaCl.

SDS-polyacrylamide gel electrophoresis (PAGE) and protein identification. The 15% Tris-glycine sodium dodecyl sulfate (SDS)-polyacrylamide gel was run at 25°C with 25 mA per gel. Protein silver staining was applied according to the method of Heukeshoven and Dernik (9). For protein identification, the protein solution was concentrated with natriumdesoxycholate-trichloroic acid precipitation by factor 30. Gels were run for 10 min at 25 mA per gel to remove remains of unpolymerized acrylamide and *N,N,N',N'*-tetramethylethylenediamine before separating proteins. After the separation, protein bands were detected using mixed Coomassie staining. Excised bands were digested with 12×10^{-6} µg liter⁻¹ trypsin at 37°C for 12 h and identified with an Ultraflex I matrix-assisted laser desorption-ionization time-of-flight/time-of-flight mass spectroscope (Bruker Daltronics, Bremen, Germany) according to the method of Schäfer et al. (23). Analysis of the peptide fragment patterns and peptide sequences derived by tandem mass spectrometry analysis was performed with the BioTools software package (Bruker Daltronics, Bremen, Germany) coupled with the Mascot software program (Matrix Science, London, United Kingdom) using the National Center for Biotechnology Information database.

Cloning. Genomic DNA from *Synechococcus* sp. strain PCC 7942 was isolated according to the method of Wu et al. (32). The *fabG* gene, coding for the 3-ketoacyl-[acyl-carrier-protein] reductase (KR) (EC 1.1.1.100) (19), was amplified by PCR (forward primer, 5'-AGAGATCCCATGGCCATGACTGCTTTGCCCCTAA-3'; reverse primer, 5'-AGAGATCGGTACCCTAGGCCATCACC AAGCC-3'). Because the genome of *Synechococcus* sp. strain PCC 7942 is completely sequenced, the primers were designed on the basis of the available *fabG* sequence (NCBI accession number CP000100; region, 678025 to 678774). *Nco*I and *Kpn*I restriction sites are underlined, and the stop codon is indicated in bold. The translational start codon of the construct was provided by the N-terminal His₆-MBP tag. The purified and digested PCR product was ligated into the pETM-41 vector, which had been previously digested with the same restriction enzymes. The resulting vector was transformed into *E. coli* XL-10 Gold. After isolation from XL-10 cells and DNA sequencing (Seqlab, Göttingen, Germany), the plasmid was transformed into *E. coli* Tuner (DE3) cells.

Recombinant overexpression and purification of the KR. A 4-ml preculture was inoculated with a single colony, grown overnight (15-ml test tubes, 37°C, 150 rpm, 16-mm excentricity) and subcultured (1:200 [vol:vol]) into 200 ml of Terrific broth (29) supplemented with 34 mg liter⁻¹ kanamycin (1,000-ml shaking flask without baffles, 37°C, 250 rpm, 5-mm excentricity). When cells had reached an optical density at 600 nm (OD₆₀₀) of 0.8, protein production was induced by adding isopropyl-β-D-thiogalactopyranoside to a final concentration of 1 mM. Thereafter, cells were cultivated at 20°C and 250 rpm for 16 h and collected by centrifugation. Cell pellets were suspended in ice-cold IMAC-A buffer (50 mM potassium phosphate [pH 8.5], 300 mM NaCl, 20 mM imidazole) at a ratio of 1 g cell (wet weight) to 5 ml IMAC-A buffer. After addition of 0.2 mM PMSF, cells were disrupted using 50% glass beads (0.25 to 0.30 mm; Braun B. Biotech, Melsungen, Germany) in a mixer mill (Retsch, Haan, Germany) for 3 min at 30 Hz. The homogenate was subsequently centrifuged at 47,808 × g, 4°C, for 30 min. After DNase digestion, the supernatant fractions were applied to a 5-ml HisTrap FF crude column (GE Healthcare, Uppsala, Sweden). The His₆-MBP-KR fusion protein was purified using standard immobilized metal affinity chromatography as described by the manufacturer. After purification, the protein solution was concentrated in a Vivaspin ultrafiltration device (molecular weight cutoff, 5,000 kDa; Sartorius Stedim Biotech, Göttingen, Germany). The His₆-MBP tag was cleaved at 4°C overnight by adding one OD₂₈₀ of TEV protease per 30 OD₂₈₀ of fusion protein. The TEV protease had previously been expressed and purified according to the method of Cherry, Tropea, and Waugh (as described at http://mcl1.ncifcrf.gov/waugh_tech.html). After cleavage, the KR was purified from the His-tagged MBP and TEV protease by passing the solution again through a 5-ml HisTrap FF crude column. The homogeneity of the purified preparation was judged by using a 15% Tris-glycine SDS-polyacrylamide gel stained with silver.

Oligomeric state. The oligomeric state of recombinantly expressed and purified KR was determined by blue-native polyacrylamide gel electrophoresis (BN-PAGE) and SEC. BN-PAGE was performed according to the method of Schägger et al. (24) using constant 13% and 20% resolving gels. Scanned images of the gels were analyzed using the program ImageJ (public domain software at <http://rsb.info.nih.gov/ij/>).

Standard proteins were obtained from Sigma (St. Louis, MO). SEC was carried out using a HiLoad 26/60 Superdex 200 column (Amersham, Uppsala, Sweden) operated at flow rates of 2 ml min⁻¹ in 20 mM Tris-HCl buffer (pH 8)–200 mM NaCl–1 mM EDTA. Proteins were detected by absorbance at 280 nm.

Enzyme assays. (i) KR assay. Unless otherwise indicated, KR activities were measured using 4 mM NADPH, 0.1 M sodium phosphate buffer, pH 7.0, and 50 to 125 mg liter⁻¹ enzyme as determined using the bicinchoninic acid assay (Pierce, Rockford, IL). KR purified from recombinant *E. coli* was used in all experiments. Due to the water immiscibility and volatile nature of the different substrates, all reactions were carried out under gas-tight conditions in 1-ml (32-by 11-mm) glass vials on a rotary shaker at 600 rpm and 30°C. After the vials had been sealed, the prochiral ketones were added through a septum, using a syringe, at different concentrations depending on the respective *K_m* value. The kinetic parameters *V_{max}* and *K_m* were estimated using nonlinear regression analysis. MATLAB R2006b (MathWorks, Massachusetts) together with the subroutine NLFIT from the Statistics Toolbox software program was used for least-squares parameter fitting on the basis of a Levenberg-Marquardt algorithm. One unit corresponds to the amount of enzyme reducing 1 µmol substrate per min at 30°C. All activities were calculated from triplicate determinations with appropriate controls run in parallel.

The influence of pH on KR activity was determined with the following 50 mM buffer systems: sodium citrate (pH 4 to 6), 2-(*N*-morpholino)ethanesulfonic acid buffer (pH 5.5 to 6.5), sodium phosphate (pH 6.5 to 8), 3-(*N*-morpholino)propanesulfonic acid (pH 6.5 to 8), Tricine (pH 7.5 to 9), and Tris-HCl (pH 8.5 to 10). All activities were estimated as percentages of the maximum.

Optimum temperature and activation energy of the KR were determined by incubating the enzyme for 5 min with 150 mM 2',3',4',5',6'-pentafluoroacetophenone at temperatures ranging from 10 to 70°C in 50 mM sodium phosphate buffer (pH 8). Phosphate buffer was chosen since it has a low temperature (*T*) dependency ($dpK_a/dT = -0.0028$, where *pK_a* is the negative decadic logarithm of the acid dissociation constant) (16). The subsequent reaction was started by adding 0.5 mM NADPH. The activation energy was calculated using the Arrhenius equation.

(ii) Alcohol dehydrogenase assay. The activity of the alcohol dehydrogenase from *Lactobacillus brevis* (LB-ADH) was determined in crude extracts of *E. coli* BL21(DE3) (pBtac-*adh_{L.brevis}*) cells. Growth and expression conditions were carried out as described by Ernst et al. (4). Crude extracts were prepared by breaking washed *E. coli* cells with glass beads as described above. After centrifugation (47,808 × g, 4°C, 30 min), activities were measured in 100 mM sodium phosphate buffer supplemented with 1 mM NADPH, 1 mM MgCl₂, and 150 mM 2',3',4',5',6'-pentafluoroacetophenone at 30°C. Protein quantifications were done by densitometry using ImageJ.

Analytics. Analysis of educts and products was performed by chiral gas chromatography using the following columns: BGB-174 (BGB-Analytics, Schlossboeckelheim, Germany) for measurement of 1,1,1-trifluoroacetone and substituted acetophenones as described previously (3, 8), BGB-175 (BGB-Analytics, Schlossboeckelheim, Germany) for 2-octanone, and Lipodex-E (Macharey-Nagel, Düren, Germany) for ethyl 4-chloroacetoacetate and ethylbenzoylacetate.

Statistics. Statistical analysis was performed using the SigmaPlot software package. The results are expressed as means ± standard deviations. The paired *t* test was used to assess statistical significance between enzyme activities of treated samples and those of untreated ones. A significance level of *P* < 0.05 was chosen.

RESULTS

Identification of the ketoreductase. The ketoreductase from *Synechococcus* sp. strain PCC 7942 responsible for the reduction of 2',3',4',5',6'-pentafluoroacetophenone was purified by gel filtration and ion exchange chromatography on the basis of its enzymatic activity. After the final chromatographic step, SDS-PAGE revealed the presence of several protein bands in the active fraction with molecular masses ranging between 10 and 50 kDa. These proteins were identified by peptide mass fingerprinting using matrix-assisted laser desorption-ionization time-of-flight tandem mass spectrometry as phosphoglyceratekinase, superoxiddismutase, 3-ketoacyl-[acyl carrier protein] reductase, and different phycobilisomal proteins. In consideration of the physiological functions of these enzymes, the 3-ketoacyl-[acyl

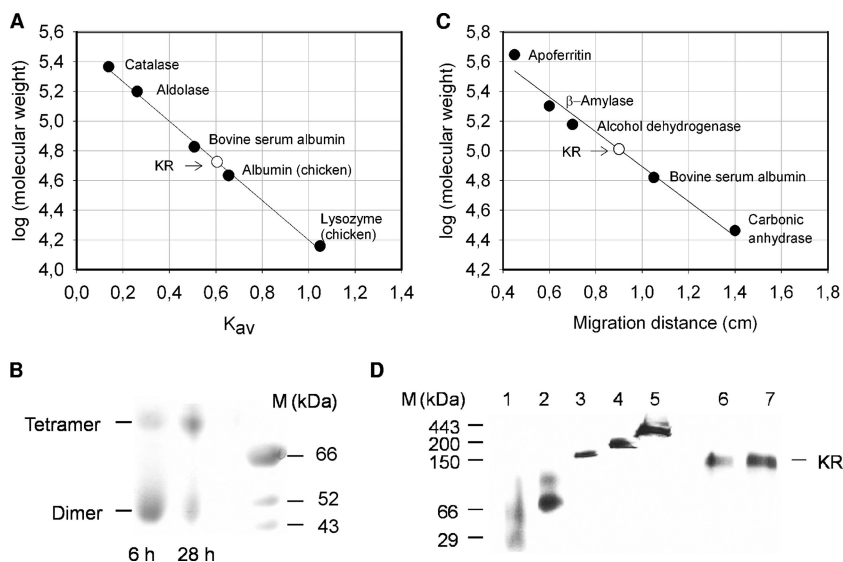


FIG. 1. Quarternary structure of the KR. (A) Calibration of the gel filtration column. Partition coefficients (K_{av}) of standard proteins (●) or KR (○) are plotted against the logarithm of the molecular weight. A mixture of the following proteins was applied as a standard: catalase (molecular weight, 232,000), aldolase (158,000), bovine serum albumin (67,000), albumin (chicken; 43,000), and lysozyme (chicken; 14,400). (B) BN-PAGE (13% resolving gel) after SEC. The protein fraction corresponding to the dimer was subjected to BN-PAGE after 6 h or 28 h of storage at 4°C. Standard proteins were bovine serum albumin (molecular mass [M], 66 kDa), α -amylase (52 kDa), and albumin (chicken; 43 kDa). (C) Molecular weight calibration curve for BN-PAGE without previous SEC. The native molecular weight markers (29,000 to 443,000) were as follows: apoferritin (443,000), β -amylase (200,000), yeast alcohol dehydrogenase (150,000), bovine serum albumin (66,000), and carbonic anhydrase (29,000). The arrow indicates the calculated size of the KR. (D) BN-PAGE (20% resolving gel) without previous SEC. Lanes 1 to 5 represent the molecular mass (M) standards. Protein amounts of KR were 5 and 10 μg (lanes 6 and 7). The arrow indicates the calculated size of the KR.

carrier protein] reductase (KR) (EC 1.1.1.100; NCBI accession number YP_399703) was the one that was most likely responsible for the reduction of prochiral ketones. It is a key enzyme in fatty acid biosynthesis, performing the first reductive step in the elongation cycle (26). Subsequent cloning and expression of the *fabG* gene in fusion with a His₆-MBP-tag led to an enzyme that exhibited the desired activity. Induced cells showed His₆-MBP-KR expression that accounted for nearly 20% of the soluble cellular protein. Typical yields of pure protein were 0.43 to 0.67 mg protein per gram dry cell weight. Since no contaminating protein was detected in the KR preparation by silver staining, the purity was judged to be near 100%.

Determination of the oligomeric state. The KR monomer has a computed molecular mass of 25.5 kDa, which was confirmed by SDS-PAGE. The quaternary structure of the enzyme was determined by two approaches.

First, we applied SEC. The data were converted into partition coefficients (K_{av}), where $K_{av} = (V_e - V_0)/(V_t - V_0)$ and V_e , V_0 , and V_t are the elution volume, void volume, and total bed volume, respectively. The results were plotted as a logarithm of the molecular mass versus K_{av} (Fig. 1A). SEC gave a single peak of protein and activity (data not shown), with an apparent molecular mass of 53 kDa corresponding to a homodimeric species. However, prior studies reported the oligomerization state of homologous enzymes, e.g., from *E. coli* (18) or rape (5), to be tetrameric, having a 222 symmetry with two types of dimerization interfaces. Therefore, the 53-kDa fraction from SEC was subjected to BN-PAGE after 6 h and 28 h of storage at 4°C (Fig. 1B). After 6 h, 85.0% of the protein was dimeric and 15.0% tetrameric. Interestingly, the percentage of tetra-

mers rose within 28 h to 72.7%, indicating a slow association equilibrium of dimers to tetramers in solution.

To further investigate the oligomer under equilibrium conditions, we investigated the size of the oligomer by BN-PAGE, omitting the SEC procedure (Fig. 1C and D). In this experiment, BN-PAGE clearly revealed tetrameric KR. The calculated molecular mass of the tetramer (102 kDa) corresponded almost exactly to the size determined for the sole protein band in the KR samples (103 kDa). Furthermore, the activity of the tetramer was 1.26 times higher than the activity of the dimeric fraction eluted from the gel filtration column. In summary, these observations indicate that under equilibrium conditions KR exists as a tetramer. The tetramer is slowly assembled noncovalently from two active dimers as revealed by the non-equilibrium SEC technique.

pH optimum. The activity of the KR as a function of the pH value and the buffer system was studied between pHs 4 and 10. The enzyme displayed a broad pH optimum over the range of 7 to 9 when zwitterionic buffers were used, with a maximum at pH 8 in Tricine buffer (Fig. 2A). Variation of the buffer molarity between 25 mM and 200 mM or addition of NaCl up to 500 mM had no significant effect on enzyme activity.

Temperature optimum. Figures 2B and C illustrate the temperature dependence of the KR activity for the reduction of 2',3',4',5',6'-pentafluoroacetophenone. The optimum temperature was found to be 44°C. Between 10 and 40°C, the activity increased, following the Arrhenius equation, with a calculated activation energy of 49.42 kJ mol⁻¹.

Inhibition study. The effect on enzyme activity of different metal ions (Ca²⁺, Co²⁺, Cu²⁺, Fe²⁺, Fe³⁺, K⁺, Mg²⁺,

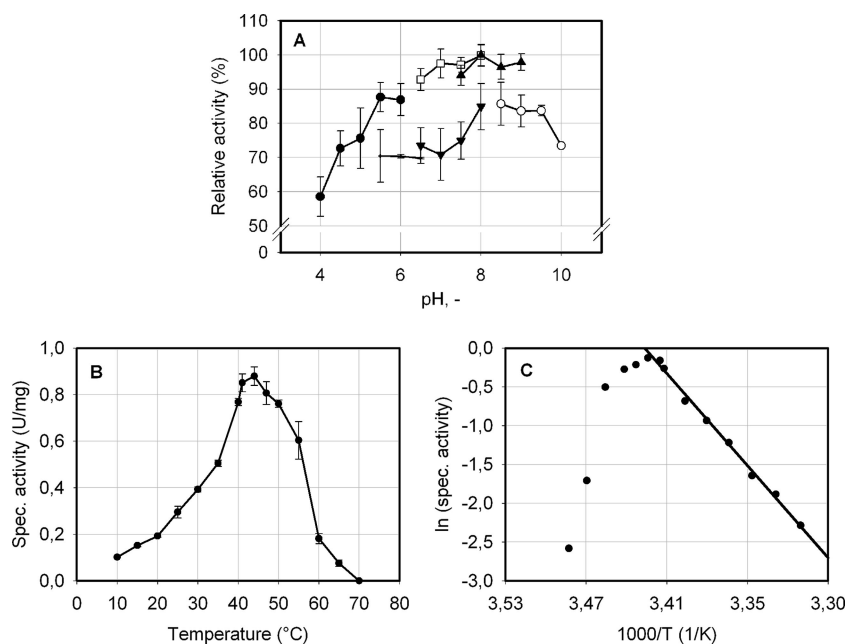


FIG. 2. Determination of pH and temperature optima. Means and standard deviations were obtained from triplicates. (A) pH dependence of KR activity. Buffers (50 mM) used: ●, Na citrate; —, 2-(*N*-morpholino)ethanesulfonic acid; ▼, Na phosphate; □, 3-(*N*-morpholino)propanesulfonic acid; ▲, Tricine; ○, Tris-HCl. (B) Effect of temperature on the specific activity (Spec. activity) of the KR. (C) Arrhenius plot of the data shown in panel B for determination of the activation energy.

Mn²⁺, and Zn²⁺) was investigated at final concentrations of 1 mM and 10 mM. The enzyme tolerated a wide range of metal ions, except for Fe²⁺ and Fe³⁺, both of which inhibited the enzyme activity significantly ($P < 0.05$). Reducing agents like β -mercaptoethanol and dithiothreitol also inhibited the enzyme ($P < 0.05$), whereas the metal chelating reagent EDTA and the protease inhibitor PMSF did not significantly alter enzyme activities.

Substrate specificity. The cyanobacterial fatty acid elongation cycle has an absolute requirement for NADPH as a cofactor (27). For this reason, the K_m for NADPH was investigated in a sub-

strate excess experiment with 2',3',4',5',6'-pentafluoroacetophenone. The K_m was determined to be 0.40 ± 0.03 mM. In order to study the substrate specificity of the purified enzyme, different ketones were tested with NADPH in excess. The results are summarized in Table 1. In general, the reduction took place following Prelog's rule, i.e., (*S*)-alcohols were formed (17). The enzyme followed Michaelis-Menten kinetics with all substrates, and it was found that the KR is efficient in reducing aromatic and aliphatic ketones. As expected, the enzyme displayed high activity toward 2',3',4',5',6'-pentafluoroacetophenone. This substrate was reduced to the corresponding (*S*)-alcohol at optimal reaction con-

TABLE 1. KR substrate specificities and enantioselectivities for the reduction of different prochiral ketones under standard reaction conditions

Substrate	Sp act (U mg ⁻¹)	Relative activity (%) ^a	K_m (mM)	Enantiomeric excess (%)
Acetophenone and derivatives				
Acetophenone	0.13 ± 0.01	100	46.92 ± 7.64	>99.0 (<i>S</i>) ^b
α -Chloroacetophenone	2.45 ± 0.09	1,885	2.71 ± 0.44	87.6 ± 1.5 (<i>R</i>)
4'-Chloroacetophenone	0.15 ± 0.00	115	14.34 ± 1.48	79.5 ± 2.5 (<i>S</i>)
3'-Fluoroacetophenone	0.13 ± 0.00	100	7.38 ± 1.16	79.9 ± 0.8 (<i>S</i>)
4'-Fluoroacetophenone	0.08 ± 0.00	62	12.29 ± 0.24	77.8 ± 1.4 (<i>S</i>)
2',3',4',5',6'-Pentafluoroacetophenone	3.93 ± 0.14	3023	5.72 ± 0.93	>99.8 (<i>S</i>) ^b
Propiophenone	0.16 ± 0.01	123	6.03 ± 1.20	>99.4 (<i>S</i>) ^b
β-Ketoesters				
Ethyl 4-chloroacetoacetate	38.29 ± 2.15	29,454	8.31 ± 1.20	99.8 ± 0.4 (<i>S</i>)
Ethylbenzoylacetate	1.16 ± 0.04	892	1.92 ± 0.24	>99.7 (<i>S</i>) ^b
Aliphatic ketones				
2-Octanone	0.02 ± 0.00	15	ND ^c	ND
1,1,1-Trifluoroacetone	1.03 ± 0.10	792	89.2 ± 17.8	47.7 ± 1.7 (<i>S</i>)

^a In relation to the specific activity obtained with acetophenone.

^b No *R* enantiomer detectable.

^c ND, not done.

ditions (44°C, pH 8, Tricine buffer), with a maximum activity of $8.57 \pm 0.49 \text{ U mg}^{-1}$. Under standard reaction conditions (30°C, pH 7, sodium phosphate buffer), the KR was sevenfold more active than the LB-ADH ($3.93 \pm 0.14 \text{ U mg}^{-1}$ versus $0.56 \pm 0.08 \text{ U mg}^{-1}$), thereby producing a significantly higher enantiomeric excess (100% [S] versus 43.3% [S]). The highest activity observed was obtained with ethyl 4-chloroacetoacetate, which was reduced with $38.29 \pm 2.15 \text{ U mg}^{-1}$ and 99.8% enantiomeric excess.

DISCUSSION

The KR from *Synechococcus* sp. strain PCC 7942 displayed biochemical characteristics similar to those observed for the functionally homologous enzyme from *E. coli*, which has temperature and pH optimum around 45°C and pH 8, respectively (28). However, the catalytic properties of the *E. coli* enzyme are different from those of the *Synechococcus* protein. For example, the FabG homologue from *E. coli* was shown to convert ethyl 4-chloroacetoacetate to the corresponding (S)-alcohol with an enantiomeric excess that is 6.1% lower than that for the protein from *Synechococcus* sp. strain PCC 7942 (99.8% versus 93.7%) (30). The same reaction was also studied using the FabG protein from *Bacillus subtilis*, which likewise exhibited a lower stereoselectivity. It reduced ethyl 4-chloroacetoacetate with an enantiomeric excess of 98.0% (30).

Determination of the oligomeric state by BN-PAGE indicated that the KR exists as a tetramer under equilibrium conditions. The reassociation of dimers to tetramers after the nonequilibrium SEC technique and the slightly reduced activity of the dimeric protein suggest that one of the putative dimer-dimer interactions of the KR was disrupted by the SEC procedure. Interestingly, the structural studies of Wickramasinghe et al. (31) of its homologue, FabG, from *Plasmodium falciparum* led to exactly the same results: a dimeric/trimeric structure was predicted by the nonequilibrium SEC technique, whereas another, equilibrium-based method—analytical ultracentrifugation—clearly indicated a tetramer. In consideration of these observations, we suppose that the KR from *Synechococcus* sp. strain PCC 7942 forms a tetramer in solution, similar to homologous enzymes that have been structurally characterized so far (5, 12, 18, 31).

On closer examination of the substrate spectrum, it is remarkable that the KR had comparatively low activity with aliphatic ketones like 2-octanone. In comparison to the natural substrates of the enzyme, this compound is just lacking an additional ester function. The results obtained for the reduction of halogenated acetophenones were consistent with the data presented for whole-cell biotransformations with *Synechococcus* sp. strain PCC 7942 (13, 14): the perfluorinated acetophenone was accepted much better than *para* and *meta* monosubstituents. Moreover, the reduction of the *para*-chlorinated acetophenone resulted in a higher activity than the reduction of the *para*-fluorinated molecule. The introduction of a halogen atom at the α position of the side chain enhanced KR activity but reduced enantioselectivity.

A comparison between the substrate spectra of LB-ADH and cyanobacterial KR reveals that the KR is capable of overcoming limits of industrially applied enzymes in the synthesis of halogenated chiral alcohols like (S)-(pentafluorophenyl) ethanol. If the activity toward acetophenone is taken as a

reference, then both enzyme activities behave contrarily. Compared to acetophenone, aromatic compounds with bulky side chains like propiophenone and ethylbenzoylacetate were catalyzed slowly by LB-ADH (10). In contrast, the KR performed better with those sterically demanding substrates than with the reference. Furthermore, β -ketoesters, such as ethyl 4-chloroacetoacetate, which strongly resembles the natural substrates of the KR, were reduced with high activities if catalyzed by the KR, whereas the LB-ADH showed poorer activities on those substrates relative to that with acetophenone (10).

The data presented in this study indicate that enzymes from cyanobacteria could fill some of the present serious gaps in the landscape of biocatalytic synthesis of optically pure alcohols. The ketoreductase from *Synechococcus* sp. strain PCC 7942 is just one member of a huge pool of uncharacterized enzymes from cyanobacteria, which represent one of the greatest subgroups of gram-negative bacteria (20). The results of a homology search for the protein sequence of the KR (NCBI accession number YP_399703) in nonredundant databases using the BLASTP algorithm (2) identified more than 40 cyanobacterial proteins with sequence identities greater than 75%. Therefore, the characterization of the KR from *Synechococcus* sp. strain PCC 7942 was the first step toward revealing the potential of cyanobacterial KRs for asymmetric syntheses (D. Weuster-Botz, J. Havel, and K. Hölsch, November 2007, International patent application PCT/EP2007/004465).

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