Rational engineering of 2-deoxyribose-5-phosphate aldolases for the biosynthesis of (R)-1,3-butanediol

Received for publication, October 4, 2019, and in revised form, December 4, 2019 Published, Papers in Press, December 5, 2019, DOI 10.1074/jbc.RA119.011363

Taeho Kim‡¶, ‡ Peter J. Stogios§, ‡ Anna N. Khusnutdinova§, ‡ Kayla Nemr§, ‡ Tatiana Skarina§, ‡ Robert Flick§, ‡ Jeong Chan Joo*, ‡ Radhakrishnan Mahadevan‡, ‡ Alexei Savchenko‡, ‡ and ‡ Alexander F. Yakunin‡¶ From the ‡Department of Chemical Engineering and Applied Chemistry, University of Toronto, Toronto, Ontario M5S 3E5, Canada, the ¶Center for Bio-Based Chemistry, Division of Convergence Chemistry, Korea Research Institute of Chemical Technology, Yuseong-gu, Daejeon 34114, Korea, the **Department of Microbiology, Immunology, and Infectious Diseases, University of Calgary, Calgary, Alberta T2N 1N4, Canada, the ¶¶Centre for Environmental Biotechnology, School of Natural Sciences, Bangor University, Bangor LL57 2UW, United Kingdom, and the ¶¶¶Future Technology Center, LG Chem, Gangseo-gu, Seoul 150-721, Korea

Edited by F. Peter Guengerich

Carbon–carbon bond formation is one of the most important reactions in biocatalysis and organic chemistry. In nature, aldolases catalyze the reversible stereoselective aldol addition between two carbonyl compounds, making them attractive catalysts for the synthesis of various chemicals. In this work, we identified several 2-deoxyribose-5-phosphate aldolases (DERAs) having acetaldehyde condensation activity, which can be used for the biosynthesis of (R)-1,3-butanediol (1,3BDO) in combination with aldo-keto reductases (AKRs). Enzymatic screening of 20 purified DERAs revealed the presence of significant acetaldehyde condensation activity in 12 of the enzymes, with the highest activities in BH1352 from Bacillus halodurans, TM1559 from Thermotoga maritima, and DeoC from Escherichia coli. The crystal structures of BH1352 and TM1559 at 1.40–2.50 Å resolution are the first full-length DERA structures revealing the presence of the C-terminal Tyr (Tyr224 in BH1352). The results from structure-based site-directed mutagenesis of BH1352 indicated a key role for the catalytic Lys155 and other active site residues in the 2-deoxyribose-5-phosphate cleavage and acetaldehyde condensation reactions. These experiments also revealed a 2.5-fold increase in acetaldehyde transformation to 1,3BDO (in combination with AKR) in the BH1352 F160Y and F160Y/M173I variants. The replacement of the WT BH1352 by the F160Y or F160Y/M173I variants in E. coli cells expressing the DERA + AKR pathway increased the production of 1,3BDO from glucose five and six times, respectively. Thus, our work provides detailed insights into the molecular mechanisms of substrate selectivity and activity of DERAs and identifies two DERA variants with enhanced activity for in vitro and in vivo 1,3BDO biosynthesis.

The formation of carbon–carbon bonds via aldol condensation of two carbonyl compounds is indispensable in biological systems and organic chemistry (1–3). Aldol condensation reactions generate a new β-hydroxy carbonyl compound, which is a valuable precursor in the construction of complex organic molecules caused by the formation of up to two new stereogenic centers (4). Using aldehydes as donor substrates in aldol reactions is particularly of interest because this provides the opportunity for sequential aldol condensation reactions to synthesize more complex molecules (5, 6). In biological systems, aldolase enzymes catalyze the reversible and stereoselective aldol addition of a nucleophilic donor onto an electrophilic aldehyde acceptor (7). The formation of a new C–C bond is accompanied by the generation of a new stereocenter, making aldolases attractive tools in the synthesis of chiral compounds and bioactive molecules. Therefore, aldolases have emerged as a promising alternative in the biocatalytic synthesis of rare sugars and sugar derivatives, such as statins, iminocyclitols, epothilones, and sialic acids (8–10).

2-Deoxyribose-5-phosphate aldolases (DERA, E.C. 4.1.2.4)2 are found in all kingdoms of life and represent the major aldolase group. One of the best-characterized DERAs is the Escherichia coli DeoC, which belongs to the class I (metal-independent) aldolases (9, 10). The E. coli deoC is part of the deo operon (deoABCDE) involved in the utilization of extracellular deoxyribose-5-phosphate as energy sources (11). It transforms the d-2-deoxyribose-5-phosphate (DRP) intermediate into d-glyceraldehyde-3-phosphate and acetaldehyde, which enter glycolysis and the Krebs cycle, respectively (12). The DERA reaction is reversible, because it also catalyzes the aldol condensation between acetaldehyde (the donor molecule) and d-glyceraldehyde-3-phosphate (the acceptor molecule) producing DRP (Scheme 1) (13). This class of aldolases is unique in that it can catalyze the aldol condensation of two aldehydes and does not require a ketone substrate, whereas other aldolases use ketones as aldol donors and aldehydes as acceptors (10, 14). It activates

2 The abbreviations used are: DERA, 2-deoxyribose-5-phosphate aldolase; 1,3BDO, (R)-1,3-butanediol; 3HB, 3-hydroxybutanal; AKR, aldo-keto reductase; DRP, 2-deoxyribose-5-phosphate; GDH, glyceraldehyde-3-phosphate dehydrogenase; PDC, pyruvate decarboxylase; TPI, triosephosphate isomerase; RMSD, root-mean-square deviation; TIM, triosephosphate isomerase; PDB, Protein Data Bank; vvm, vessel volume/min.

This is an Open Access article under the CC BY license.

© 2020 Kim et al. Published under exclusive license by The American Society for Biochemistry and Molecular Biology, Inc.

J. Biol. Chem. (2020) 295(2) 597–609 597
the donor molecule (acetaldehyde) via the catalytic Lys residue, forming a covalent Schiff base intermediate (enamine) followed by the carboligation between the acceptor (>glyceraldehyde-3-phosphate or second acetaldehyde) and the Schiff base (14, 15). The crystal structure of the E. coli DERA (DeoC) adopts the ubiquitous triosephosphate isomerase (TIM) barrel (α/β)8 fold with the catalytic Lys167 (the Schiff base-forming residue) located on strand β6 (16). A proton relay system composed of Asp102, Lys201, and a water molecule is involved in shuffling a proton between C2 of the acetaldehyde imine and enamine and subsequent C3 hydroxyl protonation. In addition, several biochemical studies suggested that the C-terminal Tyr259 of the E. coli DeoC is crucial for enzyme activity (16–18). However, all published crystal structures of DERA show the absence of electron density for the last eight C-terminal residues including Tyr (16, 19–22). Recently, using a combination of NMR spectroscopy and molecular dynamics simulations, it has been shown that the C-terminal Tyr259 of the E. coli DeoC enters the active site in catalytically relevant closed states and is required for efficiency of the proton abstraction step of the DERA catalytic reaction (18).

The acetaldehyde-active DERAs are also distinguished by their ability to ligate three aldehyde molecules in a sequential and stereoselective manner, making them attractive biocatalysts for synthetic organic chemistry (23, 24). It has been shown that DERA catalyzes a sequential tandem aldol reaction of chloroacetaldehyde and two acetaldehyde molecules, forming (3R,5S)-6-chloro-2,4,6-trideoxyhexopyranoside. This product can be further used as a lactone moiety for the synthesis of 3-hydroxy-3-methylglutaryl–CoA reductase inhibitors, cholesterol-lowering statin drugs (e.g. atorvastatin and rosuvastatin) (25–31). Thus, DERA enzymes offer the great potential by providing an effective and simplified route for their production.

Recently, we established a novel pathway to produce (R)-1,3-butanediol (1,3BDO) from acetaldehyde using DERA as the key enzyme (32, 33). The non-natural diol 1,3BDO is used as a building block for the production of synthetic polymers, pharmaceuticals, fragrances, insecticides, and antibiotics (34–39). Presently, 1,3BDO has been produced mainly from petroleum-based feedstocks using chemical processes, which require harsh reaction conditions and release toxic intermediates and by-products (38). Therefore, the development of biocatalytic processes for the production of 1,3BDO from renewable feedstocks is of increasing importance (39, 40). The recently proposed artificial biosynthetic approach for 1,3BDO production from glucose is based on a reversed fatty acid β-oxidation pathway, which includes four heterologous enzymes and requires three NADPH and one CoA molecules per molecule of 1,3BDO produced (41, 42). In contrast, the proposed DERA-based pathway for 1,3BDO production involves three heterologous enzymes: pyruvate decarboxylase (PDC), producing acetaldehyde from pyruvate), DERA (catalyzing aldol condensation of two acetaldehyde molecules to 3-hydroxybutanal), and aldoketo reductase (AKR), which reduces 3-hydroxybutanal (3HB) to 1,3BDO (Scheme 2). The heterologous expression of this pathway in E. coli resulted in the production of 0.3 g of 1,3BDO/liter from glucose (11.2 mg/g of glucose) (33). Using a systems metabolic engineering approach, the 1,3BDO titer was increased to 2.4 g/liter and yield was increased to 56 mg/g of glucose, further highlighting the potential of aldolases for synthesis of valuable products. This study also suggested that the rate-limiting step of the proposed 1,3BDO pathway is the DERA-catalyzed aldol condensation of acetaldehyde to 3HB (33).

Although our recent studies revealed great potential of DERAs for biocatalytic conversion of acetaldehyde to 1,3BDO (33), this activity (acetaldehyde condensation) has not been examined in depth. The scarcity of data on DERAs limits our efforts on increasing the acetaldehyde condensation activity of these enzymes, which represents the rate-limiting step in the biocatalytic synthesis of 1,3BDO and potentially statin drugs. In this work, after screening 20 purified microbial DERAs, we identified BH1352 from the alkalophilic bacterium Bacillus halodurans, as well as TM1559 from Thermotoga maritima and E. coli DeoC as the most active aldolases in the DERA-AKR–coupled production of 1,3BDO from acetaldehyde. The crystal structures of these enzymes were determined including the first full-length DERA structure (BH1352) and revealed the catalytic residues and substrate-binding sites. Using structure-based site-directed mutagenesis, we identified the BH1352 residues critical for acetaldehyde condensation and designed several DERA variants with higher activity in the production of 1,3BDO both in vitro (from acetaldehyde) and in vivo (from glucose). We demonstrated that E. coli cells expressing the DERA-AKR pathway with engineered DERA variants produced 5–6 times more 1,3BDO from glucose compared with cells with the WT BH1352.

Results and discussion

Phylogenetic analysis of DERA sequences

To provide insight into the phylogenetic diversity of DERAs, 2,553 sequences of putative DERAs were extracted from the Kyoto Encyclopedia of Genes and Genomes Orthology database using the identifier K01619 for the E. coli DERA (DeoC), which is the best-characterized DERA enzyme (23). Initially, this pool of putative DERA proteins included more than 2,500 sequences (2,281 from bacteria, 120 from archaea, and 152 from eukaryotes), but it was reduced to 1,974 proteins after removing redundant sequences. This phylogenetic analysis revealed the presence of five major clusters of DERA proteins including one bacterial domain, one Firmicutes (Bacilli and Clostridia), one mostly Proteobacteria, and two mixed clusters.

---

**Scheme 1.** Reversible retro-aldol reaction catalyzed by DERA.

**Scheme 2.** DERA-based pathway for 1,3BDO production from pyruvate.
To screen DERAs for the bioconversion of acetaldehyde to 1,3BDO, we selected 20 DERA proteins from different phylogenetic groups, which were found to be soluble when expressed in *E. coli* (Fig. S1 and Table S1). Based on the phylogenetic analysis, 17 selected DERAs belong to the five large clusters (clusters 1–5), whereas the remaining three proteins were from nonclassified sequences.

**Figure 1. Phylogenetic analysis of DERAs and screening of purified proteins for 1,3BDO formation.**

A, phylogenetic analysis of the DERA family: unrooted phylogenetic tree of 2,553 DERA sequences showing the presence of five main clusters (clusters 1–5) and nonclustered sequences. Black circles indicate the 20 DERA proteins from different clusters selected for activity screening (with organism names and UniProt codes). BH1352 from *B. halodurans* characterized in this work is indicated by the red circle. B, screening of 20 purified DERAs for the production of 1,3BDO from acetaldehyde in the presence of PA1127. The graph bars represent the final concentrations of 1,3BDO produced after 2 h of incubation with 10 mM NADPH and 50 mM acetaldehyde (see “Experimental procedures” for experimental details).

**Screening of purified DERAs for biosynthesis of 1,3BDO from acetaldehyde**

In our previous work, we identified several aldo-keto reductases (AKRs) with significant activity in reducing 3-hydroxybutanal to 1,3BDO (Scheme 2) (32). From these proteins, PA1127 from *Pseudomonas aeruginosa* was found to exhibit negligible activity against acetaldehyde, making it suitable for coupling of
the DERA-catalyzed condensation of acetaldehyde (to 3-hydroxybutanal) with the AKR-catalyzed reduction of 3-hydroxybutanal to 1,3BDO (Scheme 2). Using PA1127, we established a coupled enzyme system (DERA + PA1127) (Fig. S2) and screened 20 purified DERAs for transformation of acetaldehyde to 1,3BDO. These screens revealed significant production of 1,3BDO in the presence of 12 DERAs with the highest activity observed in TM1559 from *T. maritima* (DERA group 1), *E. coli* DeoC (DERA group 4), and BH1352 from *B. halodurans* (DERA group 2) (Fig. 1). Because BH1352 was found to support the highest production of 1,3BDO by *E. coli* cells expressing different DERAs (including TM1559 and DeoC) (33), this protein was selected for detailed structural and biochemical studies of the transformation of acetaldehyde to 1,3BDO.

Because *B. halodurans* is an alkaliophilic bacterium (grows well at pH >9.0), we determined the optimal pH range for BH1352 using the retro-aldol DRP cleavage reaction coupled with glyceraldehyde-3-phosphate dehydrogenase and triose-phosphate isomerase (16, 24, 43, 44). These assays revealed a broad pH range with the maximal activity of BH1352 at pH 7.2–9.5 (Fig. S3), whereas the previously reported DERA enzymes from other bacteria showed the highest activity at pH 6.0–7.5 (24, 28). At optimal pH, *V* \(_{\text{max}}\) of BH1352 with DRP as substrate was calculated to be 52–67 μmol/min/mg protein, which is lower than that for the DERA from *Lactobacillus brevis* (102 μmol/min/mg protein) but higher than other DERAs (0.25–1.00 μmol/min/mg protein) (24, 28). The *L. brevis* DERA has also been reported to exhibit high resistance to aldehydes, but this enzyme showed low activity in the AKR-based screen for 1,3BDO synthesis (Fig. 1B) (28). Steady-state kinetic parameters of BH1352 and its variants were also determined using the DRP cleavage reaction (Table 1). These experiments revealed that BH1352 exhibits typical Michaelis–Menten kinetics with the apparent *K* \(_{\text{m}}\) = 0.22 mM, which is close to that for *E. coli* DeoC and more than 10 times lower than that for the *L. brevis* DERA (3.3 mM) (24).

### Crystal structures of DERAs: overall fold and active site

The crystal structures of BH1352 (PDB codes 6D33 and 6MSW) and *E. coli* DeoC (PDB code 1KTN) were determined to 2.50 and 1.40 Å resolution, respectively, using the sitting-drop vapor diffusion method (Table 2), whereas the unpublished crystal structure of TM1559 is available from Protein Data Bank (PDB codes 3R12 and 3R13, Joint Center for Structural Genomics). In contrast to *E. coli* DeoC, the structures of BH1352 and TM1559 revealed the presence of electron density for the C-terminal Tyr residues (Tyr\(^{224}\) and Tyr\(^{246}\), respectively) making them the first full-length DERA structures. In BH1352, Tyr\(^{224}\) is located on a flexible strand, whereas the TM1559 Tyr\(^{246}\) is located on the C-terminal α-helix (Fig. 2 and Fig. S4). Analysis of crystal contacts of BH1352 using the quaternary prediction PDBePISA server (http://www.ebi.ac.uk/pdbe/pisa/)\(^{3}\) predicted a dimeric state (Fig. 2A). This was supported by the result of size-exclusion chromatography, suggesting that this protein exists as a dimer in solution (observed molecular mass, 51.7 kDa; predicted mass of monomer molecule, 24.2 kDa) (Fig. S5). It is similar to the dimeric state of hyperthermophilic and *L. brevis* DERAs but is different from the *E. coli* DeoC, which was found to exist in a monomer–dimer equilibrium (Fig. S6) (21, 24). Based on the BH1352 structure, the interfaces between monomers in each adjacent dimer involve 13 hydrogen bonds and burial 1,288 Å\(^2\) surface area. The dimerization interface of BH1352 is composed mainly of hydrophobic interactions including the BH1352 loops containing Pro\(^{134}\), Phe\(^{201}\), Pro\(^{207}\), Leu\(^{208}\), Ile\(^{207}\), and Phe\(^{160}\) (Table S2), although the weak dimerization interface of DeoC (573 Å\(^2\)) is comprised of a single hydrogen bond and two salt bridges in between α3 and α4 helices of each protomer (Fig. S6) (21, 24, 45). On the other hand, the TM1559 structure exhibits stronger dimerization interactions with the interface 1,464 Å\(^2\) between TM1559 protomers including 14 hydrogen bonds and two salt bridges along with many hydrophobic contacts, which is in line with higher structural stability of this protein (Table S2) (24).

The monomeric structures of BH1352, TM1559, and DeoC displayed a classical (α/β)\(_n\) fold (TIM barrel), which is one of the most common protein folds catalyzing diverse enzymatic reactions (46). Interestingly, the AKR enzyme PA1127 used in combination with DERAs (EC 4.1.2.4) for 1,3BDO synthesis also has a TIM barrel fold but catalyzes the NADPH-dependent reduction of 3HB and other aldehydes (EC 1.1.1.1.X) (32). A Dali search for structural homologues of BH1352 identified several DERA structures as the best matches, including the *Entamoeba histolytica* DeoC (PDB code 3NG); Z score, 39.0; root-mean-square deviation (RMSD), 0.9 Å; 63% sequence identity), *Streptococcus suis* DERA (PDB code 5DBU; Z score, 38.5; RMSD, 0.6 Å; 66% sequence identity), and *L. brevis* DERA E78K mutant (PDB code 4XBS; Z score, 38.1; RMSD, 0.9 Å; 53% sequence identity).

Based on the BH1352 structure, its active site is located inside of the β-barrel, near its C-terminal side (Fig. 2B). The active site entrance is formed by the several loops connecting β-strands (β1, β6, and β7) with α-helices (α1, α6, and α7), containing highly or semiconserved residues including Thr\(^{12}\), Leu\(^{14}\), Lys\(^{15}\), Phe\(^{66}\), Ile\(^{128}\), Phe\(^{160}\), Ser\(^{18}\)\(^{18}\), and Ser\(^{209}\). The side chains of these residues create a narrow channel providing access of substrates to the catalytic Lys\(^{15}\), located on the β6 strand (Fig. 3A). In the best-characterized DERA from *E. coli*, the catalytic Lys\(^{167}\) is in close proximity to the side chains of conserved Lys\(^{137}\) and Lys\(^{201}\), and the three Lys residues form salt bridges.

### Table 1

| Protein | *K* \(_{\text{m}}\) (mM) | *V* \(_{\text{max}}\) (μmol/min/mg) | *k* \(_{\text{cat}}\) (s\(^{-1}\)) | *k* \(_{\text{cat}}/K*_{\text{m}}\) (μmol/min/mg) |
|---------|-----------------|-----------------|-----------------|-----------------|
| WT      | 0.22 ± 0.02     | 34.1 ± 1.0      | 13.3 ± 0.4      | 60.4            |
| F160Y   | 0.50 ± 0.05     | 47.8 ± 1.6      | 18.6 ± 0.6      | 37.2            |
| F160H   | 0.45 ± 0.04     | 32.9 ± 1.1      | 12.8 ± 0.4      | 28.5            |
| F160K   | 0.06 ± 0.01     | 9.1 ± 0.4       | 3.5 ± 0.2       | 58.8            |
| F160M   | 0.17 ± 0.01     | 22.6 ± 0.2      | 8.8 ± 0.2       | 51.7            |
| F160W   | 0.22 ± 0.01     | 19.1 ± 0.4      | 7.5 ± 0.2       | 33.9            |
| I170V   | 0.18 ± 0.01     | 38.2 ± 0.5      | 14.9 ± 0.2      | 82.5            |
| M173I   | 0.17 ± 0.02     | 30.1 ± 0.8      | 11.7 ± 0.2      | 68.9            |
| M173L   | 0.21 ± 0.02     | 32.8 ± 1.1      | 12.8 ± 0.4      | 60.8            |
| M173V   | 0.18 ± 0.02     | 25.4 ± 0.9      | 9.9 ± 0.3       | 54.9            |
| F160Y/M173I | 0.25 ± 0.02 | 27.9 ± 0.7 | 10.9 ± 0.3 | 43.5           |

3 Please note that the JBC is not responsible for the long-term archiving and maintenance of this site or any other third party hosted site.
with the side chain oxygens of conserved Asp\textsuperscript{102} (16, 21). During the DERA-catalyzed synthesis of 2-deoxyribose-5-phosphate (DRP) (Scheme 1), the uncharged nucleophilic Lys\textsuperscript{167} of \textit{E. coli} DeoC attacks the acetaldehyde carbonyl forming a carbinolamine and then a Schiff base, which subsequently tautomerizes to an enamine group and attacks glyceraldehyde 3-phosphate (16, 43). Finally, hydrolysis of the aldol condensation intermediate produces the free enzyme and DRP.

Table 2

| Structure | apo BH1352 | apo BH1352 K184L | EcDERA |
|-----------|------------|------------------|--------|
| PDB code  | 6D33       | 6MSW             | 1KTN   |
| Data collection |            |                  |        |
| Space group | C2         | C2               | C2     |
| Cell dimensions |            |                  |        |
| a, b, c (Å) | 240.91, 55.52, 177.71 | 240.78, 55.13, 176.30 | 62.57, 53.56, 81.36 |
| α, β, γ (°) | 90, 128.02, 90 | 90, 127.71, 90 | 90, 109.97, 90 |
| Resolution, Å | 30.0–2.50 | 30.0–2.17 | 50.0–1.40 |
| R\textsubscript{merge} | 0.049 (0.654) | 0.077 (0.734) | 0.078 (0.309) |
| R\textsubscript{free} | 0.027 (0.392) | 0.042 (0.405) | — |<sup>c</sup> |
| CC\textsubscript{1/2} | 0.750 | 0.815 | — |<sup>c</sup> |
| Complete, % | 98.9 (97.5) | 100 (100) | 99.5 (98.2) |
| Redundancy | 3.9 (3.5) | 4.2 (4.2) | 6.0 (4.5) |
| Resolution, Å | 30.02–2.50 | 29.98–2.17 | 38.65–1.40 |
| No. of unique reflections: working, test | 63,680, 1,995 | 97,396, 1,997 | 88731, 4,458 |
| R factor/free R factor<sup>e</sup> | 20.4/25.0 (29.7/32.9) | 19.3/21.1 (28.8, 30.0) | 18.6/20.5 (18.7/19.9) |
| No. of refined atoms, molecules | Protein 9,557, 6 | Solvent 116, 18 | Solvent 97,396, 1,997 |
| | Water 461 | Water 102, 17 | Water 843 |
| | Protein 74.70 | Protein 56.58 | Protein 8.54 |
| B-factors | Solvent 97.53 | Solvent 85.32 | Solvent 54.92 |
| | Water 64.25 | Water 631 |
| | RMDS | 0.004 | 0.004 |
| | Bond angles, ° | 0.632 | 1.300 |
| | Bond lengths, Å | 0.618 | 0.004 |

<sup>a</sup> R\textsubscript{sym} = \sum_i I_i(h) / \sum_i I(h), where I_i(h) is the observed intensity of reflection h.

<sup>b</sup> The figures in parentheses indicate the values for the outer shells of the data.

<sup>c</sup> Value not measured.

<sup>d</sup> Value refers to the outer shells of the data.

<sup>e</sup> r = \sum (F\textsubscript{obs} – F\textsubscript{calc})^2 / \sum F\textsubscript{calc}, where F\textsubscript{obs} and F\textsubscript{calc} are the observed and calculated structure factor amplitudes, respectively.

**Figure 2.** Crystal structures of DERAs: BH1352. **A**, dimeric structure of BH1352. **B**, overall view of BH1352 protomer. The α-helices and β-strand structures that compose the TIM barrel are indicated and labeled. The catalytic triad is displayed with sticks.
Interestingly, the deletion of Tyr 259 (100-fold reduction of the DRP cleavage activity (16, 18). Relevant closed states, where Tyr259 is inserted into the active disordered with the equilibrium between open and catalytically has been shown that the DeoC C-terminal tail is intrinsically increased the DeoC activity in the condensation reaction combination of NMR and molecular dynamics simulations, it shows the predicted binding mode of 2-deoxyribose-5-phosphate (DRP) in the active site of BH1352. The model was generated using structural superimposition of crystal structures of BH1352 and the EcDERA–DRP complex (PDB code 1JCL) (16). This revealed the BH1352 active site residues and suggested their role in substrate binding and catalysis.

Because the catalytic residues of E. coli DeoC are conserved in BH1352 and TM1559, the same catalytic mechanism can be applied to aldol condensation of two acetaldehyde molecules catalyzed by these enzymes. In the BH1352 active site, the side chain of conserved Asp92 (Lys179 in TM1559) forms salt bridges with the conserved Lys126 (2.7 Å from Asp92), Lys155 (3.1 Å), and Lys184 (3.0 Å). We propose that the catalytic Lys155 forms a Schiff base with the acetaldehyde carbonyl, whereas Asp92 and Lys184 are part of the BH1352 proton relay system involved in imine deprotonation to form an enamine (Fig. S7). This is consistent with the results of site-directed mutagenesis of BH1352 with the respective mutant proteins (D92A, K126A, K155A, and K184A) showing very low or no catalytic activity both in the DRP cleavage and in acetaldehyde condensation reactions (Fig. 5). This is also supported by the crystal structure of TM1559 in complex with citrate and glycerol (PDB code 3R12), indicating that its active site includes Asp117, Lys150, Lys179 (catalytic), and Lys208 (Fig. S8). Another crystal structure of TM1559 (PDB code 3R13) also revealed the presence of additional electron density in the active site representing an unknown ligand covalently bound to the catalytic Lys179 (likely representing one of the reaction intermediates).

To identify other BH1352 residues involved in substrate binding, we modeled DRP into the BH1352 active site using the structure of the DeoC–DRP complex of DeoC from E. coli (Fig. 3B) (16, 43). The produced model of DRP binding in the BH1352 active site predicts that the side chain of Thr12 appears to be involved in substrate coordination via hydrogen bonding with the β-hydroxyl group of DRP, as well as with Lys184 and a water molecule (Wat26; Fig. 3B). This is consistent with the results of site-directed mutagenesis, which revealed that Ala replacement of Thr12 resulted in a catalytic impairment in the DRP cleavage reaction (Fig. 5A). However, the T12A mutant protein exhibited acetaldehyde condensation activity comparable with that of the WT BH1352, suggesting that this residue is not critical for acetaldehyde condensation (Fig. 5B).

The DRP-binding model also suggested that the DeoC Lys172 (interacting with the DRP phosphate and γ-hydroxyl groups) is replaced by Phe (Y259F) resulted in a ~100-fold reduction of the DRP cleavage activity (16, 18). Interestingly, the deletion of Tyr259 (ΔY259) significantly increased the DeoC activity in the condensation reaction between acetaldehyde and chloroacetaldehyde (27). Using a combination of NMR and molecular dynamics simulations, it has been shown that the DeoC C-terminal tail is intrinsically disordered with the equilibrium between open and catalytically relevant closed states, where Tyr259 is inserted into the active site close to the catalytic Lys167 (~ 6 Å) (18). Remarkably, the structures of both BH1352 and TM1559 revealed the presence of electron density for the C-terminal Tyr224 and Tyr246, structures of both BH1352 and the Tim55 of TM1559 (PDB code 3R13), indicating that its active site includes Asp117, Lys150, Lys179 (catalytic), and Lys208 (Fig. S8). Another crystal structure of TM1559 (PDB code 3R13) also revealed the presence of additional electron density in the active site representing an unknown ligand covalently bound to the catalytic Lys179 (likely representing one of the reaction intermediates).
densation reactions (Fig. 5), indicating that this residue plays an
important role in catalytic activity of this enzyme.

Another notable feature of the BH1352 and TM1559 structures is the presence of a cluster of hydrophobic residues near the catalytic Lys (Lys155 in BH1352), including four residues conserved in all DERAs (Leu14, Val66, Phe66, and Ile128 in BH1352 and Leu40, Val88, Phe91, and Ile152, and Phe184 in TM1559) (Fig. 6C, Fig. S8, and Table S3). In the BH1352 structure, the side chains of Leu14, Phe66, and Ile128 are oriented toward the α-carbon of aldehyde products (Fig. 6), suggesting that these residues provide hydrophobic contacts for ligand binding and that they might be essential for enzyme activity. This was supported by the results of alanine replacement mutagenesis of these residues, which produced mutant proteins with a greatly reduced activity in both reactions (the L14A protein was found to be insoluble) (Fig. 5). Another hydrophobic cluster comprising of three valine residues (Val154, Val177, and Val183), Ile170, and Met173 is located between the two β-strands (β6 and β7) and the α6 helix (in BH1352) (Fig. 6). It was previously reported that this cluster may contribute to the sequential aldol condensation, as revealed by the DeoC mutations F200I and M185V (equivalent to Val183 and Met173 in BH1352), resulting in enhanced condensation of acetaldehyde and chloroacetaldehyde (27). Also, DeoC Phe200 is replaced by Val in DERAs from T. maritima and Pyrobaculum aerophilum, both of which show higher sequential aldol condensation of acetaldehyde (24). These results suggest that reducing the size of hydrophobic side chains in this cluster might contribute to higher aldol condensation activity.

Recently, it has been shown that the C-terminal Tyr259 of the E. coli DeoC is required for the efficient proton abstraction step in the DRP cleavage reaction (18), whereas the previous work with the truncated DeoC ΔY259 protein (Tyr259 deleted) demonstrated an enhanced activity in acetaldehyde condensation with chloroacetaldehyde (27). Because the BH1352 structure suggested that the C-terminal Tyr224 might directly contribute to substrate binding or activity of this enzyme (Fig. 4), site-directed mutagenesis was also used to ascertain the role of this residue. We designed and purified four Tyr224 mutant proteins including Y224A, Y224F, ΔY224 (Tyr224 deleted), and ΔS223/ Y224 (Ser223 and Tyr224 deleted) and tested their catalytic activities in the DRP cleavage and acetaldehyde condensation (1,3BDO production) reactions. Interestingly, although the acetaldehyde condensation reactions of these mutant proteins were not affected, their retro-aldol activity was greatly reduced (especially in Y224F), indicating that Tyr224 is essential for DRP cleavage but not for acetaldehyde condensation (Fig. 5). Thus, the crystal structures of BH1352 and other DERAs from different phylogenetic groups revealed significant differences in substrate coordination and catalysis of DRP cleavage and acetaldehyde condensation.

Structure-based engineering of BH1352 for enhanced production of 1,3BDO

The crystal structures of BH1352 and TM1559 revealed that their substrate-binding pockets also include the side chain of a
semiconserved Phe (Phe\textsuperscript{160} in BH1352 and Phe\textsuperscript{184} in TM1559) (Fig. 6 and Fig. S9). This residue is conserved in most DERAs from clusters 1 (mixed group) and 2 (Firmicutes), but it is replaced by a Lys residue in Proteobacterial DERAs (cluster 4) including \textit{E. coli} DeoC (Fig. 7). In the \textit{Lactobacillus brevis} DERA (\textit{LbDERA}), the replacement of the homologous Phe\textsuperscript{163} by Tyr has been shown to result in enhanced sequential condensation of acetaldehyde and chloroacetaldehyde, probably by promoting substrate access (30). We found that the BH1352 Phe\textsuperscript{160} was not essential both for the retro-aldol (DRP cleavage) and acetaldehyde condensation reactions, because the F160A mutation had no significant effect on both reactions (Fig. 5). However, the DRP cleavage activity of BH1352 was negatively affected when Phe\textsuperscript{160} was mutated to Glu, Gln, Lys, Met, Trp, or His and slightly stimulated by mutation to Tyr (~23%) (Fig. 5A). Interestingly, the acetaldehyde condensation via BH1352 increased almost three times in the F160Y protein and was also increased in the F160E (72%) and F160H (44%) proteins (Fig. 5B and Fig. S10). In contrast, the replacement of Phe\textsuperscript{160} with Lys, Gln, Met, or Trp had no significant effect on this activity. These results suggest that similar to \textit{LbDERA} (30), the substitution of Phe\textsuperscript{160} by Tyr in BH1352 enhances acetaldehyde binding and/or condensation but has no effect on DRP cleavage (Fig. S9). Based on the BH1352 crystal structure, the hydroxyl group of Tyr\textsuperscript{160} (in F160Y) might interact with the main chain amide of conserved Lys\textsuperscript{15} (3.3 Å) located on the β1–α1 loop (Leu\textsuperscript{13}–Thr\textsuperscript{19}) near the absolutely conserved Leu\textsuperscript{14} (Fig. S9C). Our mutagenesis studies demonstrated that Lys\textsuperscript{15} is critical for catalytic activity of BH1352, whereas Leu\textsuperscript{14} is part of the hydrophobic cluster near the catalytic Lys\textsuperscript{155} (L14A mutant protein was found to be insoluble) (Figs. 5 and 6). We propose that the hydroxyl group of Tyr\textsuperscript{160} provides a stabilizing effect on the conformation of both Leu\textsuperscript{14} and Lys\textsuperscript{15} in the BH1352 active site, resulting in increased acetaldehyde condensation activity of this enzyme.

We also mutated the semiconserved residues Ile\textsuperscript{170} and Met\textsuperscript{173} of BH1352, located near the catalytic Lys\textsuperscript{155} (Fig. 6D), to examine whether the reduction or increase of their hydrophobic side chains will affect the catalytic activity of BH1352 and improve acetaldehyde condensation. Our coupled DERA-AKR assays (1,3BDO production) revealed a 40–50% increase in the production of 1,3BDO by the purified mutant proteins I170V, M173I, M173L, and M173V compared with the WT BH1352, whereas I170A showed reduced activity (Fig. 5). In contrast, the retro-aldol (DRP cleavage) activity of BH1352 was not signifi-
Engineering DERAs for the biosynthesis of 1,3BDO

Conclusions

Using a combination of purified DERAs and an aldo-keto reductase (PA1127), we have identified three microbial DERAs with high activity in the transformation of acetaldehyde to 1,3BDO. The crystal structure and site-directed mutagenesis of BH1352 provided insights into the molecular mechanisms of substrate selectivity and acetaldehyde condensation activity of DERAs. By targeting hydrophobic residues near the catalytic Lys155 of BH1352, we generated two variants of this enzyme (F160Y and F160Y/M173I) with enhanced activity in acetaldehyde condensation and 1,3BDO production. E. coli cells expressing these BH1352 variants as part of the DERA + AKR pathway produced 5–6 times more 1,3BDO from glucose compared with cells with the WT BH1352. The designed BH1352 variants can be used as a starting material for future protein engineering efforts aimed at improving the activity of DERAs and their performance in the biotechnological production of 1,3BDO and other chemicals.

Experimental procedures

Phylogenetic and sequence analyses

The phylogenetic tree was generated by retrieving 2,553 sequences from UniProt using Kyoto Encyclopedia of Genes and Genomes Orthology identifier K01619, which represents DERAs (EC 4.1.2.4) involved in the pentose phosphate pathway. The original data set was reduced to 1,974 sequences by removing redundant sequences and increasing gap-free sites using ClustalW (61). The tree was built using FastTree 2.1.5 and visualized by Interactive Tree of Life (https://itol.embl.de/) (50, 51). The DERA sequence alignment and phylogenetic analysis were conducted as described in our previous study (32). Structural images of BH1352 were prepared using PyMOL Molecular Graphics System, version 1.8 (Schrödinger, LLC).
Figure 7. Structure-based sequence alignment of DERAs active in 1,3BDO production: six DERAs from Bacilli including BH1352 (light red background), six proteobacterial DERAs (light blue background), and TM1559 (center row). The secondary structure elements derived from the structures of BH1352 and E. coli DeoC are shown above and below the alignment, respectively. Residues conserved in all proteins are shown in white type on a red background. The columns with red residues indicate the presence of more than 70% of biochemically similar residues. The catalytic residues are indicated by cyan boxes with red residue numbers, whereas the columns with black boxes and residue numbers indicate the substrate entrance residues. The residues of the hydrophobic amino acid clusters are labeled with black circles.
Purified DERA was crystallized at room temperature using the sitting-drop vapor-diffusion method using protein concentration of 10 mg/ml and reservoir solution of 0.1 M Tris-HCl (pH 8.5), 0.2 M magnesium chloride, 25% (w/v) PEG 3350, and 10 mM acetaldehyde. The crystal was cryoprotected in the same buffer supplemented with 2% PEG 200 and flash-frozen in liquid nitrogen. Diffraction data for the BH1352 apoenzyme crystal were collected at 100-K at a Rigaku home source Micromax-007 with R-AXIS IV++ detector. Diffraction data were processed using HKL3000 (53). The structure was solved by molecular replacement using Phenix Phaser and the structure of a putative aldolase (PDB code 3NGJ) (54). Model building and refinement were performed using Phenix.refine and COOT (54, 55). TLS parameterization was utilized, and B-factors were refined as isotropic. Structure geometry and validation were performed using the Phenix MolProbity tools. The dry samples were dissolved in the same volume of double-distilled H2O and analyzed using HPLC (Dionex Ultimate 3000, Thermo Scientific) equipped with an Aminex HPX-87H column, equilibrated with 5 mM H2SO4 as an eluent with a flow rate of 0.6 ml/min at 50 °C. 1,3BDO was detected using a refractive index detector (Shodex RI-101). Assay conditions were optimized by varying concentrations of DERA, AKR, and NADPH, and the optimal conditions included 100 μg/ml each of DERA and AKR and 10 mM NADPH (Fig. S2).

The kinetic parameters of purified DERA were determined using DRP cleavage reaction using a glyceraldehyde-3-phosphate dehydrogenase/triosephosphate isomerase (GDH/TPI)-coupled assay. The DERA-catalyzed retro-aldol reaction produces acetaldehyde and D-glyceraldehyde-3-phosphate, which is converted into dihydroxyacetone phosphate by TPI and further reduced by GDH consuming NADH. The detailed assay conditions were as follows: 100 mM triethanolamine buffer, pH 8.5, 0.5 mM NADH, WT or mutant DERA (1 μg/ml), TPI (11 units/ml), GDH (1 unit/ml), and DRP (from 4 μM to 4 mM) in a 200-μl reaction mixture at 30 °C. The kinetic parameters were calculated by a nonlinear regression analysis of raw data fit to the sigmoidal function using GraphPad Prism software (version 5.04 for Windows).

For the analysis of DERA resistance against acetaldehyde, a freshly prepared acetaldehyde solution (final concentration, 100 mM) was added to the incubation mixture containing 2 mg/ml of purified BH1352 (WT or mutant proteins). The incubation solution aliquots were taken and diluted for further use in a DRP cleavage assay (1 mM DRP). The activity of DERA samples was analyzed immediately after acetaldehyde addition and then at regular time intervals. The residual DERA activity was calculated by comparison with control samples without acetaldehyde (containing enzymes and buffer).

Enzyme assays
Purified DERAs were initially screened using a DERA-AKR coupled assay with 50 mM acetaldehyde as substrate in the following reaction mixture (0.2 ml): 100 mM triethanolamine buffer (pH 7.5), 10 mM NADPH, DERA (250 μg/ml), and AKR (PA1127, 250 μg/ml). The production of 1,3BDO was measured using HPLC, following 2 h of incubation at room temperature (used in DERA-catalyzed reactions) (22). The reaction samples were filtered through centrifugal filter device (10,000 cut-off, VWR) to remove enzymes and dried to get rid of residual acetaldehyde from the samples using a vacuum concentrator. The dry samples were dissolved in the same volume of double-distilled H2O and analyzed using HPLC (Dionex Ultimate 3000, Thermo Scientific) equipped with an Aminex HPX-87H column, equilibrated with 5 mM H2SO4 as an eluent with a flow rate of 0.6 ml/min at 50 °C. 1,3BDO was detected using a refractive index detector (Shodex RI-101).
culture was then used to inoculate 300 ml of modified M9 medium (without MOPS) and supplemented with 100 μg/ml of carbenicillin in the bioreactors. The pH was controlled at 7.0 by the addition of 10% NH₄OH, stirrer speed at 1,500 rpm, temperature at 37 °C, and air flow rate at 1.5 vvm. When the culture density reached A₅₆₀nm between 7 and 8, protein expression was induced by the addition of 1 mM isopropyl β-D-thiogalactopyranoside. After 30 min, the air flow rate was reduced to 0.37 vvm (25% of the initial vvm) to reduce dissolved oxygen, and 3% glucose was additionally supplemented.

Author contributions—T. K., J. C. J., R. M., A. S., and A. F. Y. conceptualization; T. K., P. J. S., K. N., and R. F. formal analysis; T. K., P. J. S., A. N. K., N. T., R. F., and J. C. J. investigation; T. K., P. J. S., A. N. K., and J. C. J. visualization; T. K., K. N., T. S., and R. F. methodology; T. K. and A. F. Y. writing-original draft; T. K., R. F., and A. F. Y. writing-review and editing; P. J. S. and R. F. validation; T. K., A. N. K., K. N., T. S., R. F., and J. C. J. investigation; T. K., R. F., P. J. S., A. N. K., Anderson, W. F., Mahadevan, R., Savchenko, A., and Yakunin, A. F. (2017) Novel aldo-keto reductases for the biocatalytic conversion of 2-deoxy-5-phosphate aldolase: subunit structure and composition of active site lysine region. Arch. Biochem. Biophys. 164, 736–742 CrossRef Medline

Schulte, M., Petrović, D., Neudecker, P., Hartmann, R., Pietruszka, J., Willbold, S., Willbold, D., and Panwalker, V. (2018) Conformational sampling of the intrinsically disordered C-terminal tail of DERA is important for enzyme catalysis. ACS Catal. 8, 3971–3984 CrossRef Medline

Cao, T. P., Kim, J. S., Woo, M. H., Choi, J. M., Jun, Y. Lee, K. H., and Lee, S. H. (2016) Structural insight for substrate tolerance to 2-deoxy-5-phosphate aldolase from the pathogen Streptococcus suis. J. Microbiol. 54, 311–321 CrossRef Medline

Dick, M., Weiergräber, O. H., Clasen, T., Bisterfeld, C., Bramski, J., Gohlke, H., and Pietruszka, J. (2016) Trading off stability against activity in extremophiles aldolases. Sci. Rep. 6, 17908 CrossRef Medline

Heine, A., Luz, J. G., Wong, C. H., and Wilson, I. A. (2004) Analysis of the class I aldolase binding site architecture based on the crystal structure of 2-deoxyribose-5-phosphate aldolase at 0.99Å resolution. J. Mol. Biol. 343, 1019–1034 CrossRef Medline

Dick, M., Hartmann, R., Weiergräber, O. H., Bisterfeld, C., Clasen, T., Schwarten, M., Neudecker, P., Willbold, D., and Pietruszka, J. (2016) Mechanism-based inhibition of an aldolase at high concentrations of its natural substrate acetaldehyde: structural insights and protective strategies. Chem. Sci. 7, 4492–4502 CrossRef Medline

Gjøsen, H. J. M., and Wong, C.-H. (1994) Unprecedented asymmetric aldol reactions with three aldolase substrates catalyzed by 2-deoxy-5-phosphate aldolase. J. Am. Chem. Soc. 116, 8422–8423 CrossRef

Sakuraba, H., Yoneda, K., Yoshihara, K., Satoh, K., Kawakami, R., Uto, Y., Tsuge, H., Takashashi, K., Hori, H., and Ohshima, T. (2007) Sequential aldol condensation catalyzed by hyperthermophilic 2-deoxy-d-ribose-5-phosphate aldolase. Appl. Environ. Microbiol. 73, 7427–7434 CrossRef Medline

Liu, J., and Wong, C. H. (2002) Aldolase-catalyzed asymmetric synthesis of novel pyranose synthons as a new entry to heterocycles and epothilones. Angew. Chem. Int. Ed. Engl. 41, 1404–1407 CrossRef Medline

Greenberg, W. A., Varvax, A., Hanson, S. R., Wong, K., Huang, H., Chen, P., and Burk, M. J. (2004) Development of an efficient, scalable, aldolase-catalyzed process for enantioselective synthesis of statin intermediates. Proc. Natl. Acad. Sci. U.S.A. 101, 5788–5793 CrossRef Medline

Jennewein, S., Schürmann, M., Wolberg, M., Hörler, I., Luiten, R., Wubbolts, M., and Mink, D. (2006) Directed evolution of an industrial biocatalyst: 2-deoxy-d-ribose 5-phosphate aldolase. Biotechnol. J. 1, 537–548 CrossRef Medline

Jiao, X.-C., Pan, J., Xu, G.-C., Kong, X.-D., and Xu, J. H. (2017) Protein engineering of 2-deoxyribose-5-phosphate aldolase to achieve higher productivity in Lactobacillus plantarum. J. Bacteriol. 199, 347–365 Medline

Barbas, C. F., Wang, Y. F., and Wong, C. H. (1990) Deoxyribose-5-phosphate aldolase as a synthetic catalyst. J. Am. Chem. Soc. 112, 2013–2014 CrossRef

Pricer, W. E., Jr., and Horecker, B. L. (1960) Deoxyribose aldolase from Lactobacillus plantarum. J. Biol. Chem. 235, 1292–1298 CrossRef

Heine, A., DeSantis, G., Luz, J. G., Mitchell, M., Wong, C. H., and Wilson, I. A. (2001) Observation of covalent intermediates in an enzyme mechanism at atomic resolution. Science 294, 369–374 CrossRef Medline

Hoffee, P., Snyder, P., Sushak, C., and Jargiello, P. (1974) Deoxyribose-5-P aldolase: subunit structure and composition of active site lysine region. Arch. Biochem. Biophys. 164, 736–742 CrossRef Medline

Acknowledgments—We thank all members of the BioZone Centre for Applied Bioscience and Bioengineering for help in conducting experiments.

References
1. Mahrwald, R. (ed.) (2004) Model Aldol Reactions, Wiley-VCH, Weinheim, Germany

2. Mahrwald, R. (1999) Diastereoselection in Lewis-acid–mediated aldol additions. Chem. Rev. 99, 1095–1120 CrossRef Medline

3. Mukaiyama, T. (1982) The directed aldol reaction. Organic Reactions 28, 203–331

4. Windle, C. L., Müller, M., Nelson, A., and Berry, A. (2014) Engineering aldolases as biocatalysts. Curr. Opin. Chem. Biol. 19, 25–33 CrossRef Medline

5. Orsini, F., Pelizzoni, F., Forte, M., Sisti, M., Bombieri, G., and Benetollo, F. (1989) Behaviour of amino acids and aliphatic aldehydes in dipolar aprotic solvents: formation of oxazolidinones. J. Heterocycl. Chem. 26, 837–841 CrossRef

6. Mukherjee, S., Yang, J. W., Hoffmann, S., and List, B. (2007) Asymmetric enamine catalysis. Chem. Rev. 107, 5471–5569 CrossRef Medline

7. Ma, H., Szelér, K., Kamerlin, S. C. L., and Widersten, M. (2016) Linking coupled motions and entropic effects to the catalytic activity of 2-deoxyribose-5-phosphate aldolase (DERA). Chem. Sci. 7, 1415–1421 CrossRef Medline

8. Clapés, P., Fessner, W. D., Sprenger, G. A., and Samland, A. K. (2010) Recent progress in stereoselective synthesis with aldolases. Curr. Opin. Chem. Biol. 14, 154–167 CrossRef Medline

9. Machajewski, T. D., and Wong, C. H. (2000) The catalytic asymmetric aldol reaction. Angew. Chem. Int. Ed. Engl. 39, 1352–1375 CrossRef Medline

10. Haridas, M., Abdelraheem, E. M. M., and Hanefeld, U. (2018) 2-Deoxyribose-5-phosphate aldolase (DERA): applications and modifications. Appl. Microbiol. Biotechnol. 102, 9959–9971 CrossRef Medline

11. Lomax, M. S., and Greenberg, G. R. (1968) Characteristics of the deo operon: role in thymine utilization and sensitivity to deoxyribonucleosides. J. Bacteriol. 96, 501–514 Medline

12. Racker, E. (1952) Enzymatic synthesis and breakdown of deoxyribose phosphate. J. Biol. Chem. 196, 347–365 Medline

13. Valentin-Hansen, P., Boetius, F., Hammer-Jespersen, K., and Svendsen, I. (1982) The primary structure of Escherichia coli K12 2-deoxyribose 5-phosphate aldolase: nucleotide sequence of the deoC gene and the amino acid sequence of the enzyme. Eur. J. Biochem. 125, 561–566 CrossRef Medline

14. Barbas, C. F., Wang, Y. F., and Wong, C. H. (1990) Deoxyribose-5-phosphate aldolase as a synthetic catalyst. J. Am. Chem. Soc. 112, 2013–2014 CrossRef
