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Altering the Substrate Specificity of Reductase *Cg*KR1 from *Candida glabrata* by Protein Engineering for Bioreduction of Aromatic α-Keto Esters

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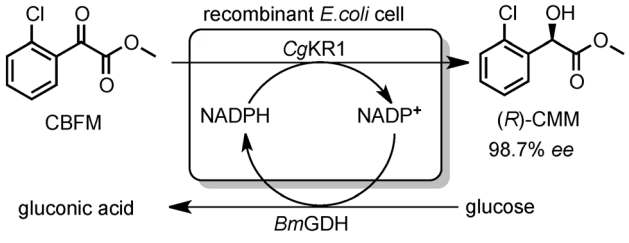
Abstract: A versatile keto ester reductase *Cg*KR1, exhibiting a broad substrate spectrum, was obtained from *Candida glabrata* by genome data mining. It showed the highest activity toward an aliphatic β-keto ester, ethyl 4-chloro-3-oxobutanoate (COBE), but much lower activity toward bulkier α-keto esters with an aromatic group, such as methyl *ortho*-chlorobenzoylformate (CBFM) and ethyl 2-oxo-4-phenylbutyrate (OPBE). By rational design of the active pocket, the substrate specificity of the reductase was significantly altered and this tailor-made reductase showed a much higher activity toward aromatic α-keto esters (~7-fold increase in k_{cat}/K_m toward CBFM) and lower activity toward aliphatic keto esters (~12-fold decrease in k_{cat}/K_m toward COBE). Meanwhile, the thermostability of the reductase was enhanced by a consensus approach. Such improvements may yield practical catalysts for the asymmetric bioreduction of these aromatic α-keto esters

Keywords: aromatic α-keto esters; protein engineering; reductases; substrate specificity

Chiral alcohols are frequently required as important intermediates for the introduction of chiral centers into the pharmaceuticals, flavors, aroma and agricultural chemicals, and specialty materials.[1] Enantioselective ketone reduction is a reliable, scalable and straightforward route to optically active alcohols. Biocatalysts are becoming preferred for ketone reduction and the application of reductases in the commercial synthesis of chiral alcohols has undergone a revolution over the past several years.[2] Furthermore, protein engineering methods have been applied to generate biocatalysts with higher activity, improved thermostability and/or better selectivity to strengthen the advantages of biocatalysts and to extend their application in the chemical and pharmaceutical industries.[3]

Recently, we discovered a versatile keto ester reductase from *Candida glabrata* (*Cg*KR1), which exhibited a broad substrate spectrum.[4] It showed the highest activity (114 U/mg protein) toward ethyl 4-chloro-3-oxobutanoate (COBE, **10**, as shown in Figure 2). In contrast, when the substrates were aromatic α-keto esters with a bulky phenyl group, such as methyl *ortho*-chlorobenzoylformate (CBFM, **1**) (Scheme 1) and ethyl 2-oxo-4-phenylbutyrate (OPBE, **4**), the activity of *Cg*KR1 decreased obviously by nearly one order of magnitude. In order to enhance the activity of *Cg*KR1 toward the synthesis of these useful chiral alcohols, a rational engineering strategy was adopted to construct NDT libraries[5] of amino acid residues located at the substrate binding site.

Given the absence of crystallographic data, three-dimensional models of *Cg*KR1 and Gre2p[6] were predicted by homology modeling using the crystal structures of a carbonyl reductase (*Ss*CR) from *Sporobolo-*



Scheme 1. Asymmetric reduction of methyl *ortho*-chlorobenzoylformate (CBFM) with recombinant cells of *E. coli*/p*Cg*KR1 and *Bm*GDH.

Table 1. Kinetic parameters of wild-type *Cg*KR1 and variants with substrates CBFM and COBE.

Enzyme	CBFM			COBE		
	K_m [mM]	k_{cat} [s^{-1}]	k_{cat}/K_m [$s^{-1}mM^{-1}$]	K_m [mM]	k_{cat} [s^{-1}]	k_{cat}/K_m [$s^{-1}mM^{-1}$]
WT	2.25 ± 0.31	74.0 ± 2.0	32.9	1.00 ± 0.24	310 ± 26	310
M1[a]	5.84 ± 1.05	918 ± 72	157	12.3 ± 0.8	442 ± 12	36
M2[b]	1.53 ± 0.33	56.0 ± 4.0	36.6	0.60 ± 0.08	162 ± 6.0	270
M3[c]	1.97 ± 0.28	482 ± 30	245	10.7 ± 1.6	288 ± 32	27

[a] The mutations are F92L/F94V.
[b] The mutations are I99Y/G174A.
[c] The mutations are F92L/F94V/I99Y/G174A.

myces salmonicolor.[7] In our previous work,[4] homology modeling and docking analyses were performed to gain insights into the high selectivity of the enzyme. The substrate molecule of methyl *ortho*-chlorobenzoylformate (CBFM) was docked into the pocket of the homology modeled structure. According to the proposed catalytic mechanism of the short-chain alcohol dehydrogenase,[7] the carbonyl oxygen atom of the ketone substrate forms hydrogen bonds with both Tyr and Ser residues and it is protonated from the Tyr residue, followed by the attack of a hydrogen atom from the C-4 atom of NADPH toward the carbonyl carbon atom of the substrate. In the structure of *Cg*KR1/CBFM complex (Supporting Information, Figure S1A), Ser134 and Tyr175 stabilize the substrate with hydrogen bonds. The aryl ring is embedded in a hydrophobic cavity next to the catalytic center, while the α-ester group is located in a small cavity mainly composed of F92, F94, N224 as well as NADPH. It is obvious that the substrate binding pocket of *Cg*KR1 is bigger than that of Gre2p, which might explain the higher activity of *Cg*KR1 toward CBFM. It implies that the binding pocket could be modified to change the substrate specificity, perhaps by rational design. To investigate the effect of different amino acid side chains at the three selected positions (F92, F94, N224) on *Cg*KR1 activity, each of the selected residues was replaced by 12 other residues (Phe, Leu, Ile, Val, Cys, Arg, Ser, Gly, Tyr, His, Asn, and Asp). Three single-site libraries were generated with the help of NDT degenerate primers, comprising 12 codon variants, with a balanced mixture of aliphatic and aromatic, polar and non-polar, positively and negatively charged residues.[8]

Among the three libraries, two variants, F92L and F94V, were identified, displaying higher activities toward CBFM, while there were no significant findings in the N224 library compared to the wild-type. Although the leucine substitution engendered the desired activity, the variant F92L was inhibited to some extent by the high concentration of substrate CBFM in further research ($K_m = 0.17$ mM, $K_{is} = 0.24$ mM). Nevertheless, we tried to combine the two mutations of F92L and F94V. To our surprise, this new double-mutated variant (*Cg*KR1M1) exhibited higher activity than either of the two single-mutated variants, without any substrate inhibition. To establish whether the increased activity of the mutant M1 (F92L/F94V) was caused by the enhanced binding affinity and/or the higher turnover rate of substrate, kinetic parameters for the enzyme-catalyzed reduction of CBFM were determined (Table 1). The results showed that the approximately 5-fold increase in activity (k_{cat}/K_m) of *Cg*KR1M1 resulted from the 12-fold increase in k_{cat} and the 2-fold increase in K_m. This accounts for the observed increase in specific activity of the mutant (109 U/mg protein) as compared to that of *Cg*KR1-WT (16 U/mg protein).

Although the activity of the double-mutated mutant M1 toward CBFM and OPBE had increased, its thermostability became worse. Importantly, enhancement of the enzyme's thermostability results in increased catalytic lifetime or total turnover number (TTN).[9] Thus, it is necessary to improve the thermostability of this mutated reductase. A number of protein-engineering strategies has been employed in an attempt to improve protein stability.[10] Taking advantage of the large number of available protein sequences, the semi-rational "consensus approach" is a well-established strategy to improve protein thermostability.[11] The sequences that we picked range in amino acid identity from 31% to 62% with respect to the wild-type, which represent a significantly wide scope (Supporting Information, Table S1).

From the output of the consensus analysis, six (S37T, I99Y, G174A, T153V, Y215F and H249F) of the 352 positions were distinct under a 50% consensus cut-off. The single mutants and some combined mutants were analyzed by a thermostability and activity assay (Supporting Information, Table S2).

Variant M2 (I99Y/G174A) was selected for further research considering both the thermostability and activity. Thus, M1 and M2 were combined for a quadruple-mutated mutant M3 (F92L/F94V/I99Y/G174A). Just as expected, the thermostability of M3 was enhanced as shown by the increase in T_{50}^{15} (defined as the temperature at which heat treatment for 15 min reduced the initial activity by 50%) by 2.3 °C as com-

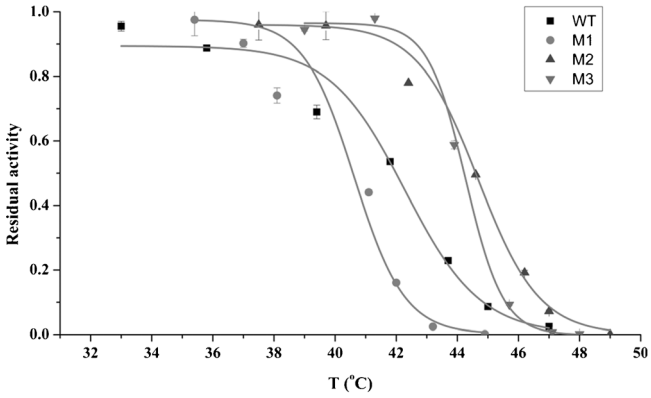


Figure 1. Thermostability of the purified WT and variants M1, M2 and M3 of *Cg*KR1 as displayed by the residual activity curves.

pared to the wide-type (T_{50}^{15}: 41.8 °C) and 3.9 °C to M1 (TT_{50}^{15}: 40.2 °C) (Figure 1). The kinetic study showed that mutant M3 exhibited a slightly lower K_m value for CBFM and ~8-fold increase in k_{cat}, when compared to those of the wide-type.

Using the purified enzyme, the substrate profile of M3 was explored in comparison with the wild-type enzyme. The activities of M3 for aliphatic keto esters were drastically lower than those of the wild-type enzyme while the activities toward all the aromatic α-keto esters tested were 3~5 times higher (Figure 2). Furthermore, substrates **1** and **10** were selected as the representatives of aromatic keto esters and aliphatic keto esters for the kinetic constants measurement. The enhanced activity of *Cg*KR1M3 for CBFM was mainly due to its higher k_{cat} value (7 times higher than that of the wild-type, Table 1). Meanwhile, the reduced activity of *Cg*KR1M3 for COBE was mainly due to its lower affinity (11-fold higher K_m than that of the wild-type, Table 1). To determine the stereoselectivity of the bioreduction by *Cg*KR1M3, 0.5 mL reactions were performed using lyophilized *Cg*KR1M3 cells in the presence of *Bm*GDH and glucose for the regeneration of NADPH.[12] Analysis of the stereoselectivity by HPLC or GC (Supporting Information, Table S4) indicated that *Cg*KR1M3 had almost the same stereoselectivity toward the majority of tested substrates except one aliphatic keto ester (substrate **6**) (Figure 2).

To demonstrate the synthetic potential of variant *Cg*KR1M3, the bioreduction of CBFM by either *Cg*KR1 wild-type or variant M3 was performed on a 100-mL scale for the preparation of methyl (*R*)-*ortho*-chloromandelate [(*R*)-CMM], a key chiral intermediate for the synthesis of (*S*)-clopidogrel. The same amount of lyophilized cells of *E. coli*/p*Cg*KR1 WT and M3 were utilized to transform CBFM at 100 g L^{-1} in 100 mL phosphate buffer with 1.5 equivalents of

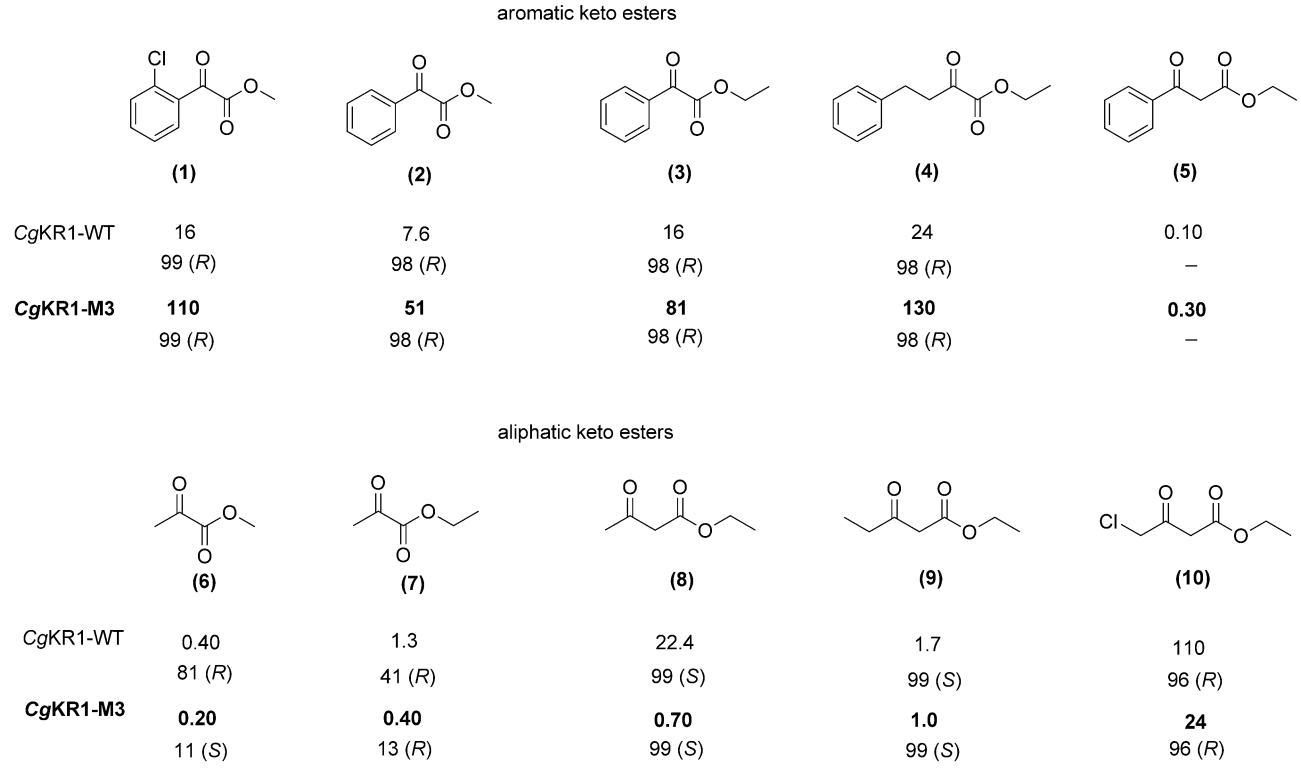


Figure 2. Specific activity (in unit of U/mg protein, upper) and stereoselectivity (% *ee*, down) of *Cg*KR1-WT and variant *Cg*KR1 M3 toward aromatic and aliphatic keto esters.

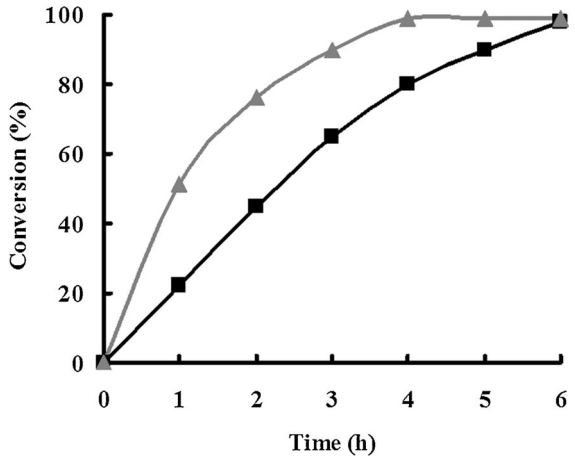


Figure 3. Progress curves of asymmetric reduction of CBFM with lyophilized cells of *E. coli*/p*Cg*KR1-WT (■) and *E. coli*/p*Cg*KR1M3 (▲). *Reaction conditions:* CBFM 100 g L^{-1}, D-glucose 1.5 equiv., lyophilized cells of *E. coli*/p*Cg*KR1 1.5 g, lyophilized *Bm*GDH powders 1.5 g, KPB 100 mL (pH 6.0, 100 mM), 25 °C. pH was kept at 6.0 with 1 M Na_2CO_3.

glucose, and lyophilized powders of *Bm*GDH (cell-free extract) was used to complete the requisite regeneration of cofactor by catalyzing the oxidation of glucose. The initial rates were relatively slow because of the impermeability of the lyophilized cells. The speeds rose after ~0.5 h along with the increased permeability. When the biocatalyst loading was 15 g L^{-1}, *Cg*KR1-WT and *Cg*KR1M3 resulted in >99% conversion within 4 h and 6 h, respectively (Figure 3). The lyophilized cells were then lowered to 7.5 g L^{-1}, in spite of the slightly longer time, *Cg*KR1M3 could transform all the substrate into optically pure (*R*)-CMM after 4.5 h while *Cg*KR1-WT only resulted in 89% conversion. When the enzyme loading was further decreased to 5 g L^{-1}, *Cg*KR1-WT and *Cg*KR1M3 gave 57% and 81% conversions, respectively (Table 2).

In order to unravel the differences in substrate specificity between *Cg*KR1M3 and *Cg*KR1-WT, we modeled the CBFM into the active sites of *Cg*KR1 WT and M3. Analysis of the most favorable docking poses of *Cg*KR1 revealed striking differences in the substrate binding pocket. The binding pocket of M3 variant (Figure 4A) is obviously larger than that of wild-type (Figure 4B). The substitution of the large amino acid F92/F94 with smaller leucine and valine apparently expands the cavity packaging the ester group, and seems to allow the substrates to adopt the catalytic conformation more easily and thus contributes to the enhanced k_{cat} observed experimentally as k_{cat} is positively related to the rate of formation of covalent intermediates,[13] which is a determinant for the increased activity.[14] Meanwhile, the significant increase in the K_m for ethyl 4-chloro-3-oxobutanoate (COBE) can contribute to the decreased activity toward aliphatic keto esters. The expanded cavity enabling the accommodation of the phenyl group may be comparatively large for the smaller aliphatic group resulting in its lower affinity (Figure 4C, D).[15] Given that both substrates **1** and **10** are converted to the corresponding *R*-enantiomers, the binding modes for **1** and **10** are supposed to be that the aromatic substituent of **1** and the ester moiety of substrate **10** occupy the same location. It suggests the different location of the ester groups of these two substrates, which gives an explanation to the much more favorable binding of the enzyme with the aromatic substrates after opening up the small binding pocket. The mutant site Ile99 is at the surface of *Cg*KR1 whereas Gly174 is in the inside of *Cg*KR1 near the surface (Figure 5). This finding underscores the important role that the protein surface plays on stability as found in many other enzymes with improved thermostability.[16] I99Y may increase the hydrogen bonds with water, or increase the hydrophilicity of the enzyme.[17] Some degree of the stabilization may due to the packing effect or the increased hydrophobic interaction as reported for

Table 2. Asymmetric reduction of CBFM with lyophilized cells of *E. coli*/p*Cg*KR1 WT and M3.[a]

Entry	Enzyme	Substrate [g L^{-1}]	Cell [g L^{-1}][b]	Time [h]	Conversion [%][c]	*ee* [%)][d]
1	WT	100	15	6	>99	98.7 (*R*)
	M3	100	15	4	>99	98.7 (*R*)
2	WT	100	7.5	5	89	98.7 (*R*)
	M3	100	7.5	4.5	>99	98.7 (*R*)
3	WT	100	5.0	10	57	98.7 (*R*)
	M3	100	5.0	10	81	98.7 (*R*)

[a] *Reaction conditions:* CBFM (100 g L^{-1}), D-glucose (1.5 equiv.), lyophilized cells of *E. coli*/p*Cg*KR1, lyophilized *Bm*GDH powders (the same amount as lyophilized cells of *E. coli*/p*Cg*KR1, in excess), 100 mL KPB (pH 6.0, 100 mM), 25 °C, pH was kept at 6.0 with 1 M Na_2CO_3.
[b] The quantity of lyophilized cells of *E. coli*/p*Cg*KR1.
[c] Determined by GC analysis.
[d] Determined by HPLC analysis.

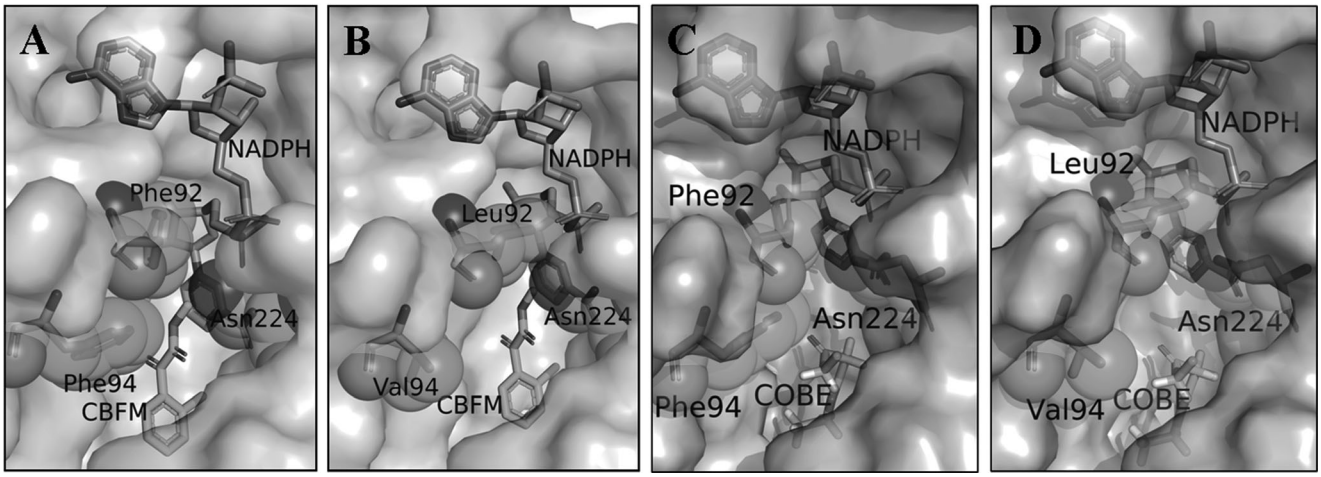


Figure 4. Molecular docking of substrates CBFM and COBE into the active site of *Cg*KR1 WT (**A**, **C**) and mutant *Cg*KR1 F92L/F94V (**B**, **D**). The cavity and crevice of *Cg*KR1 are represented by a grey surface.

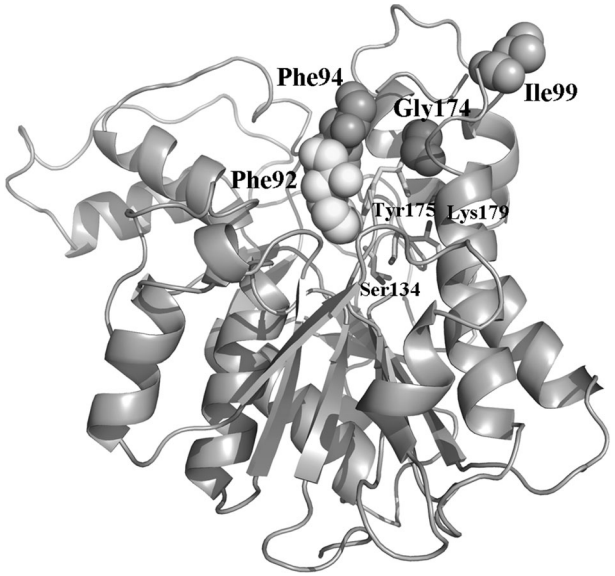


Figure 5. The model structure of *Cg*KR1. Catalytic triad: Ser/Tyr/Lys.

G174A.[18] In this case the activity and thermostability of *Cg*KR1 were enhanced by these mutations.

In summary, the activity and thermostability of *Cg*KR1 toward aromatic α-keto esters were improved by a structure-based rational design approach and consensus approach. Here we have confirmed that Phe92 and Phe94 are important in substrate recognition of *Cg*KR1 while Ile99 and Gly174 are influential residues for *Cg*KR1 thermostability. *Cg*KR1M3 displays higher activity and thermostability simultaneously toward the examined aromatic α-keto esters. Kinetic analysis and substrate docking could partially provide insights into the mechanism of the mutation effects: the mutations at positions 92 and 94 cause significant changes in the geometry of the substrate-binding pocket, which presumably facilitates the bioreduction of aromatic α-keto esters. Further improvement of the enzyme thermostability *via* protein engineering is currently underway.

Experimental Section

Generation of Mutagenesis

Mutations were introduced by PCR into the pET28-*Cg*KR1 template DNA[4] using the QuickChange (Stratagene) according to the manufacturer's instructions. The primers used in this study are listed in the Supporting Information, Table S3. Additional combination mutations were introduced in subsequent rounds of PCR. Mutagenesis was confirmed by DNA sequencing (Shanghai Sunny Biotechnology Co. Ltd, China). The mutant proteins were expressed and purified similarly as the wild-type.

T_{50}^{15} Determinations

T_{50}^{15} data were obtained by filling a row in a 96-well plate with 100 μL per well of enzyme at 1 mg mL^{-1}. The PCR plate was sealed with microseal, and a thermocycler was used to apply a temperature gradient for 15 min. The PCR plate was then immediately cooled on ice. Then, the residual activity of the enzymes was measured with the standard protocol. The T_{50}^{15} was estimated as the temperature at which heat treatment for 15 min reduced the initial activity by 50%.[19]

Consensus Approach

The sequence alignment was submitted to the online Comparative Sequence Analysis (http://coot.embl.de/Alignment/consensus.html), and the threshold was set at 50% (Supporting Information, Figure S2). Wild-type residues were mutated to the consensus amino acid if they also fit a number of criteria, such as not destroying salt bridges or helix, located in more than 6 Å from the cofactor binding site.[11a] The con-

firmed and putative or probable amino acid sequences were obtained by BLASTP programs on NCBI using amino acid sequence of *Cg*KR1 as the query and aligned with CLUSTALW software.

Acknowledgements

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