SUPPORTING INFORMATION

STRUCTURAL AND FUNCTIONAL CONSEQUENCES OF CIRCULAR PERMUTATION ON THE ACTIVE SITE OF OLD YELLOW ENZYME

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MATERIALS & METHODS

Materials: Reagents and chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless indicated otherwise. Ligase and restriction endonucleases were obtained from New England Biolabs (NEB, Ipswich, MA). *Pfu* DNA polymerase (Stratagene, La Jolla, CA) was used for the PCR. Plasmid DNA was isolated using the QIAprep Miniprep Kit and PCR products were purified with QIAquick PCR Purification Kit (Qiagen, Valencia, CA).

Gene constructs: The gene encoding OYE1 from *S. pastorianus* (formerly *S. carlsbergensis*; NCBI access number: X53597.1) served as starting sequence for our experiments. Specific circular permutated gene variants were prepared by whole-gene synthesis.(32) Variants with site-specific amino acid modifications were synthesized using the primer overlap extension method while N and C-terminal protein truncation was achieved by PCR amplification with gene-specific oligonucleotides. The genes of different variants were cloned into pET-14b (Novagen) and their correct sequences confirmed by DNA sequence analysis.

Protein Expression and Purification: Wild type OYE (OYE1), as well as OYE1 and cpOYE variants were heterologously expressed in *E. coli* and purified to homogeneity via a two-step purification protocol involving anion-exchange and gel filtration chromatography as previously described.(32) Expression levels of soluble protein for OYE1 and cpOYE variants were comparable. Individual proteins were analyzed for purity by SDS-PAGE (>95%) and for quantity by UV spectroscopy, using FMN absorbance data of the native and denatured protein.

Kinetic analysis of variants: The catalytic performance of OYE1 and cpOYE variants described in this manuscript was evaluated via reduction of two reference substrates; ketoisophorone (**1**) and (*S*)-carvone (**3**). Standard assay conditions have previously been described.(32) Briefly, experiments were performed under anaerobic conditions (McCoy chamber) in the presence of glucose/glucose dehydrogenase for NADPH cofactor regeneration. Reaction progress was monitored via chiral GC analysis.

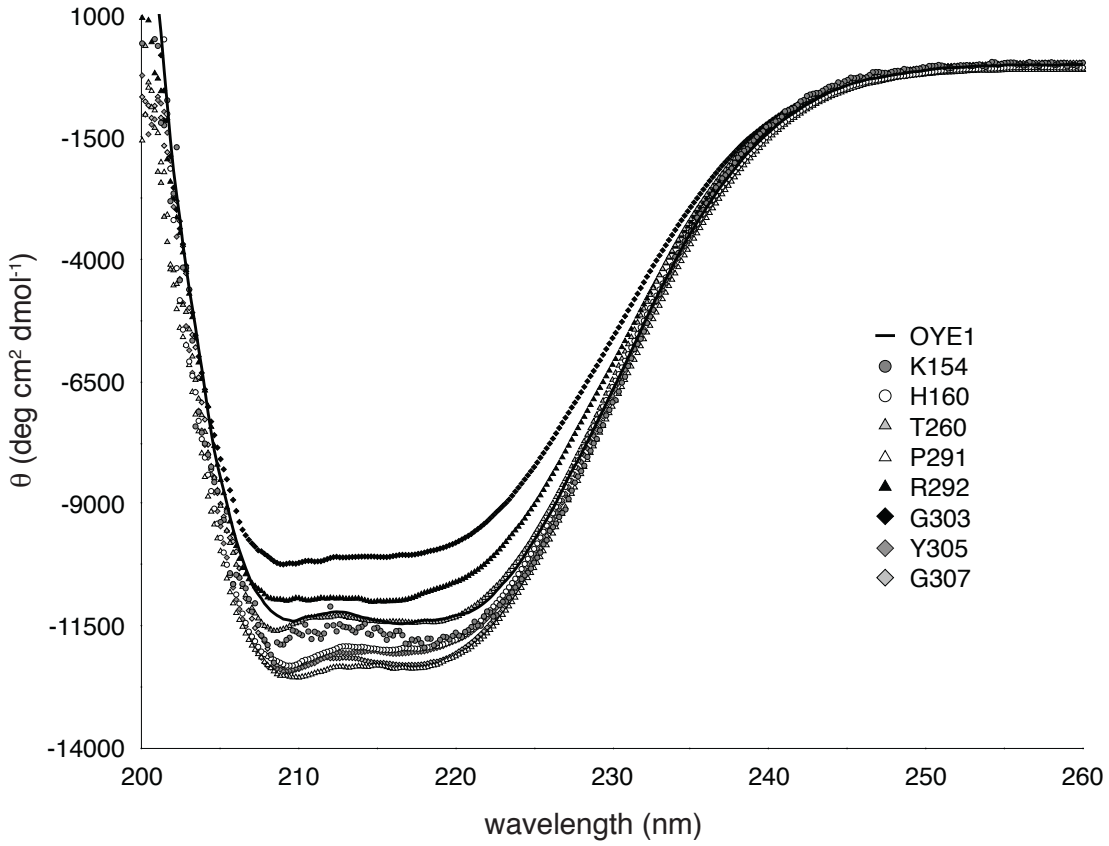
Circular Dichroism Spectroscopy: CD measurements were performed on a Jasco J-810 spectropolarimeter equipped with a Peltier unit for temperature control. All samples were prepared in 50 mM potassium phosphate buffer (pH 7.5) with protein concentrations ranging from 10 to 25 μM as determined by the absorbance at 280 nm. The spectra were collected in the far-UV range using a cuvette with a 1 mm path length at 25 °C with a scan rate of 20 nm/min, a response time of 2 s, a bandwidth of 2 nm and an average of three accumulation scans. For thermal denaturation experiments, the change in ellipticity at 222 nm was monitored from 4 to 85 °C with a temperature gradient of 1.0 °C/min and data pitch of 0.2 nm.

Determination of Oligomeric State: The oligomeric state for all enzyme variants was determined by size-exclusion chromatography on either a Superdex 200, 10/300 GL column (GE Healthcare) or a Bio SEC-3 HPLC column (Agilent, Santa Clara, CA). Protein samples (50 μM) were prepared in 40 mM Tris-HCl (pH 8.0), 300 mM NaCl. Retention times for the different oligomers were calibrated against protein standards of known molecular weight.

Crystallography: Following protein purification, sample aliquots were exchanged into protein crystallization buffer (10 mM Tris (pH8.0), 10 mM NaCl, 10 μM PMSF) and concentrated to ~40 mg/mL by ultrafiltration. Early crystallization trials, using conditions reported for wild type OYE (33), were largely unsatisfying. Hence, crystallization screens were conducted to find optimal conditions for crystallizing selected cpOYE variants by the sitting drop method at 16°C (cpOYE154: PEG 2000 MME (30%) + 0.1 M thiocyanate (pH 5.0); cpOYE303: 16-18% PEG 3350, 0.2 M $MgCl_2$, 0.1 M Tris-HCl (pH 8); cpOYE303T: 2 M ammonium sulfate, 0.1 M HEPES (pH 7.5)). Crystals typically appeared within 48 to 72 h and were acquired with a nylon loop (Hampton Research). After quickly transferring them to the well solution containing 20% ethylene glycol, they were flash-frozen directly in liquid nitrogen, stored and later used in X-ray diffraction experiments. For the structure with *p*-hydroxybenzaldehyde in the active site, protein crystals were soaked in inhibitor (11 mM) for 10 min, followed by cryoprotection.

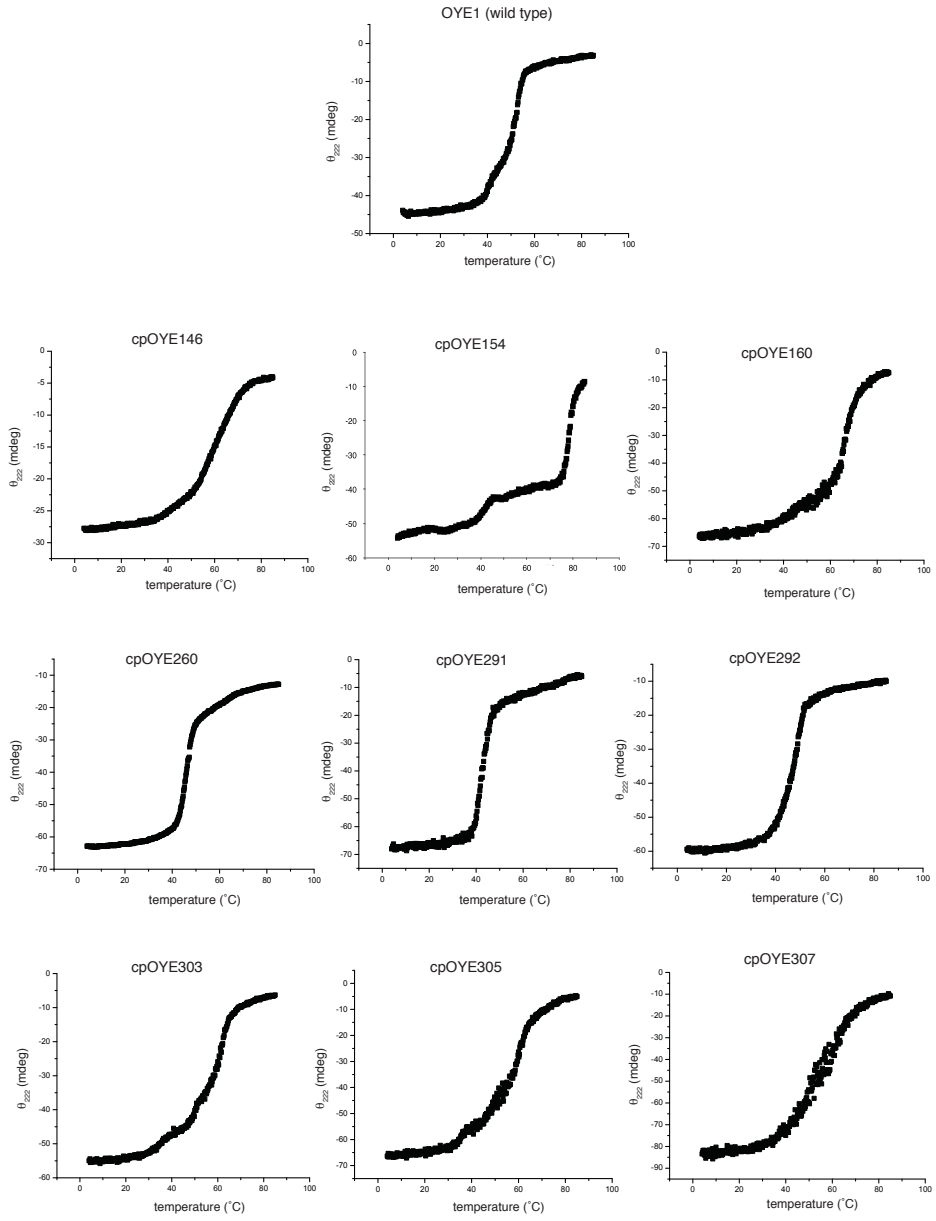
X-ray data was collected at the 22-ID and 22-BM SER-CAT synchrotron beamlines (Advanced Photon Source, Argonne National Laboratory) using their experimental control system SERGUI. The diffraction datasets were processed using the program HKL2000.(47) Phasing via molecular replacement, map production, and model refinement were conducted using the PHENIX software suite.(48) Maps and models were visualized with COOT (49), as well as by conducting manual model manipulation during refinement rounds. Molecular replacement utilized protein coordinates of the previously reported wild-type OYE1 structure (PDB code: 1OYA)(33) as the initial search model; this model was modified on the basis of evident changes in backbone connectivity of the permutants in the electron density using COOT. The coordinates for cpOYE154 (1.25Å), cpOYE303 (2.69Å), cpOYE303 with bound inhibitor (2.47Å), and truncated cpOYE303 (1.55Å) were deposited in the PDB (accession codes 4RNX, 4RNU,4RNV, and 4RNW, respectively).

Figure S1



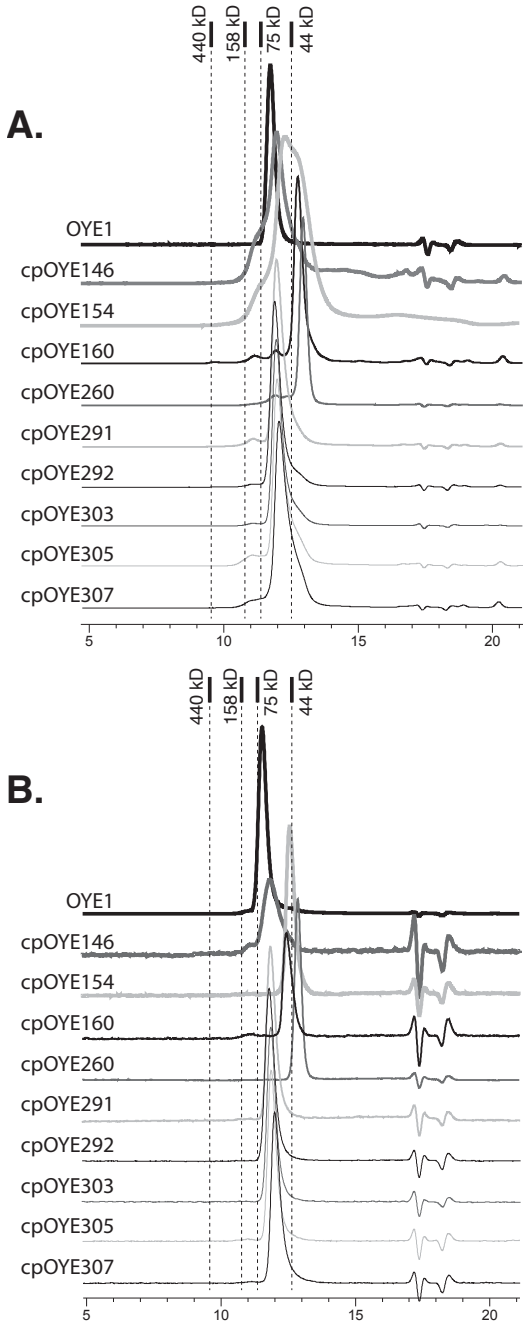
Far-UV CD spectra for native OYE1 and selected cpOYE variants. Spectra were recorded as outlined in the Material & Methods section. In all cases, the data suggest the preservation of the mixed β/α structure measured for wild type enzyme.

Figure S2



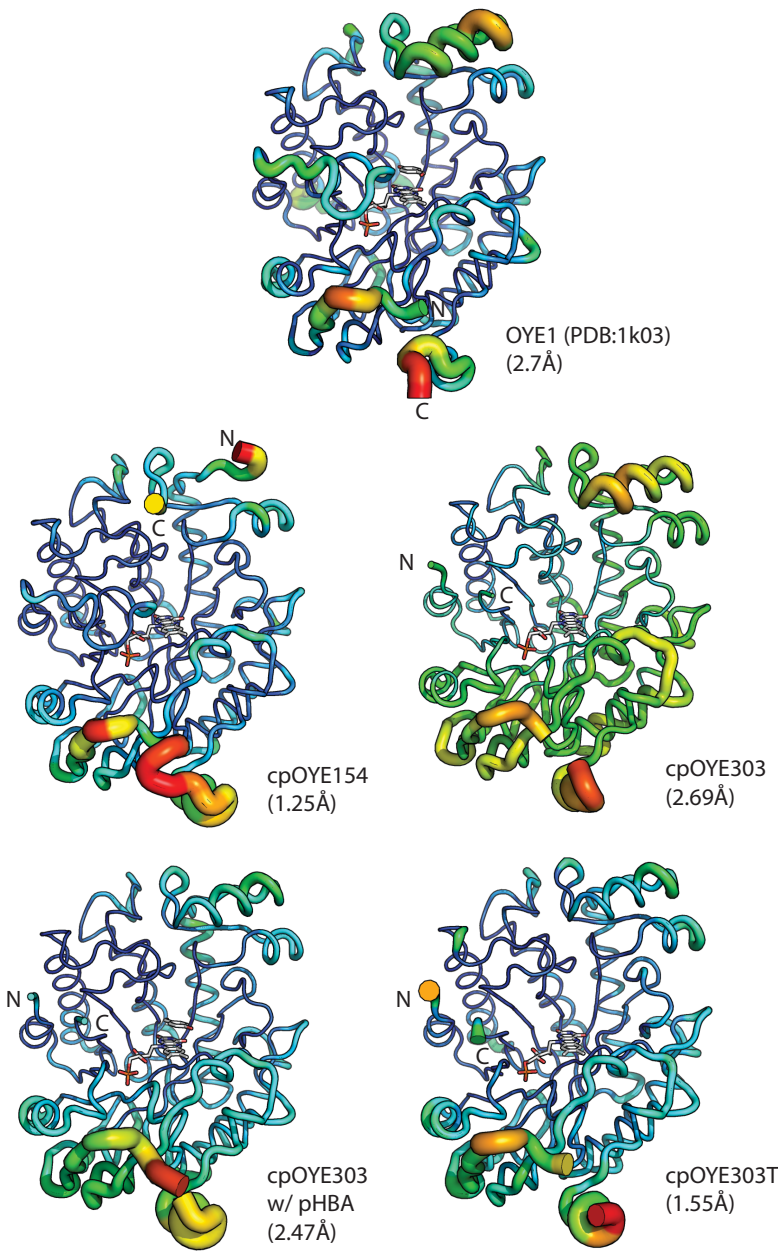
Thermodenaturation data for native OYE1 and selected cpOYE variants. The change in secondary structure at 222 nm as a function of temperature were measured in the CD spectrophotometer. The temperature of unfolding (T_M) was determined at the half point of the structure transition.

Figure S3



Size-exclusion chromatography data for native OYE1 and selected cpOYE variants. The elution time (in minutes) of target proteins, detected at 280 nm (A.) and 460 nm (B.) are shown. The retention time of size standards are marked by dashed lines.

Figure S4



Crystallographic thermal B-factor analysis for native OYE1 and selected cpOYE variants. Increased flexibility in the protein backbone is indicated by tube thickness and coloration (rigid = blue, flexible = red). Analysis was performed with the PyMol Molecular Graphics System, Version 1.6.0.0, Schroedinger, LLC.