Optimization of Enzyme Mechanism along the Evolutionary Trajectory of a Computationally Designed (Retro-)Aldolase
Supporting Information

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Figure S1. (S)-Methodol cleavage under single turnover conditions catalyzed by the original computational design RA95.0. In order to generate single turnover conditions, an excess of enzyme over substrate is required, thus 500/700 μM RA95.0 were incubated with 200 μM (S)-methodol. (S)-Methodol is the preferred substrate of RA95.0 and was therefore used in this assay. The formation of 6-MNA was monitored by fluorescence at 450 nm after excitation at 330 nm (A) or absorption at 350 nm (B). The kinetic traces were fitted with single exponential functions and the extracted single turnover rate constants were plotted against the enzyme concentration. The steady state rate constant ($k_{cat}$) equals the measured single turnover rate constants ($k_{ST}$), indicating that C-C bond scission or an earlier step in the mechanism is rate-limiting for methodol cleavage catalyzed by the computational design RA95.0.
Figure S2. Characterization of partially deuterated $d_5$-($R$)-methodol. The compound was synthesized from non-deuterated 6-MNA and fully deuterated $d_6$-acetone using RA95.5-8F as the biocatalyst as described in the Experimental Section. The characterization by mass spectrometry (A) and $^1$H-NMR spectroscopy (B) was performed in direct comparison with non-deuterated ($R$)-methodol. (A) EI-MS data show the correct masses and the fragmentation products (circles) confirm the deuteration pattern. (B) $^1$H-NMR data show that the resonances of the deuterated groups disappear completely, whereas all others remain. A list of all $^1$H and $^{13}$C chemical shifts is given in the Experimental Section.
Figure S3. Enzyme-catalyzed H/D exchange monitored by $^1$H-NMR ($^6$-acetone in D$_2$O buffer). (A) Analysis of the spin system: isotope shifts and couplings in $^6$S,$^t$-acetone. (B) $^1$H-NMR data upon incubation of 3 μM RA95.5-8F with 200 mM $^6$-acetone in D$_2$O buffer over the course of 120 min. $^3$-Acetonitrile served as the internal standard. Integrals $I_1$ (overall decreasing) and $I_2$ (increasing) were plotted over time and the respective slopes were used to calculate the H/D exchange rate constant, which was measured in duplicates and at different c(acetone) for subsequent analysis with the Michaelis-Menten equation.
Figure S4. Enzyme-catalyzed H/D exchange monitored by $^2$H-NMR ($d_6$-acetone in H$_2$O buffer). (A) Analysis of the spin system: isotope shifts and couplings in $h_1,d_5$-acetone. (B) $^2$H-NMR data upon incubation of 3 μM RA95.5-8F with 200 mM $d_6$-acetone in H$_2$O buffer over the course of 120 min. $d_3$-Acetonitrile served as the internal standard. Integrals $I_1$ (increasing) and $I_2$ (overall decreasing) were plotted over time and the respective slopes were used to calculate the H/D exchange rate constant, which was measured in duplicates and at different c(acetone) for subsequent analysis with the Michaelis-Menten equation. Note that the mono-protonated methyl group of $h_1,d_5$-acetone is represented by a doublet and integral $I_1$ only accounts for half of its area, while the growing shoulder in $I_2$ accounts for the other half.