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Supporting information for article:

Crystal structures and kinetic studies of a laboratory evolved aldehyde reductase explain the dramatic shift of its new substrate specificity

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Table S1. Crystallization and crystal treatment protocols

Data set (cocrystallization; cryoprotectant)	D93 (L259V) (with Fe^{2+}, NADH; glycerol)	DA1472 (N151G/L259V) (with Fe^{2+}, NADH; glycerol)	DA1472 (N151G/259V) (with Fe^{2+}, NAD^+, substrate analog (compound **7**); PEG600)	DA1472 (N151G/L259V) (with Fe^{2+}, NADH, substrate analog (compound **7**); PEG600)
PDB entry	7QLG	7QNH	7QLQ	7QLS
Protein buffer	10.03 mg/ml dissolved in 20 mM Tris-HCl, pH 7.5 that was supplemented with 0.5 mM $FeCl_2$ and 10 mM NADH and incubated for 10 min at room temperature.	9.25 mg/ml dissolved in 20 mM Tris-HCl, pH 7.5 that was supplemented with 0.5 mM $FeCl_2$ and 10 mM NADH and incubated for 10 min at room temperature.	9.25 mg/ml dissolved in 20 mM Tris-HCl, pH 7.5 that was supplemented with 1 mM substrate analog 0.5 mM $FeCl_2$ and 10 mM NAD^+ and incubated for 10 min at room temperature.	9.25 mg/ml dissolved in 20 mM Tris-HCl, pH 7.5 that was supplemented with 1 mM substrate analog 0.5 mM $FeCl_2$ and 10 mM NADH and incubated for 10 min at room temperature.
Well solution buffer	50.64 mM sodium acetate trihydrate, pH 4.5; 15 % (w/v) PEG 3350; 200 mM sodium formate.	50.64 mM sodium acetate trihydrate, pH 4.5; 15 % (w/v) PEG 3350; 200 mM sodium formate.	0.1 M bis-tris pH 5.5; 20 % (w/v) PEG 6000; 0.2 M sodium formate.	0.1 M sodium acetate pH 4.5; 20 % (w/v) PEG 8000; 0.2 M sodium formate.
Drop size	0.4 µL + 0.4 µL	0.4 µL + 0.4 µL	0.4 µL + 0.4 µL	0.4 µL + 0.4 µL
Crystal soaking protocol (time, composition)[a]	The crystal was transferred in 1 µL well solution, supplemented with 10 mM NADH and diluted with glycerol to a final concentration of 30 % (v/v), for 1 min.	The crystal was transferred in 1 µL well solution, supplemented with10 mM NADH, and diluted with glycerol to a final concentration of 30 % (v/v), for 1 min.	The crystal was transferred in 1 µL well solution, supplemented with10 mM NAD^+ + 1 mM compound **7** and diluted with PEG600 to a final concentration of 20 % (w/v), for 1 min.	The crystal was transferred in 1 µL well solution, supplemented with10 mM NADH + 1 mM compound **7** and diluted with PEG600 to a final concentration of 20 % (w/v), for 1 min.
Sample name (in IceBear[b])	FUCO_96ehE08d2c5	FUCO_96ekE08d1c1	FUCO_96j2G10d2c2	FUCO_96j1F06d3c1

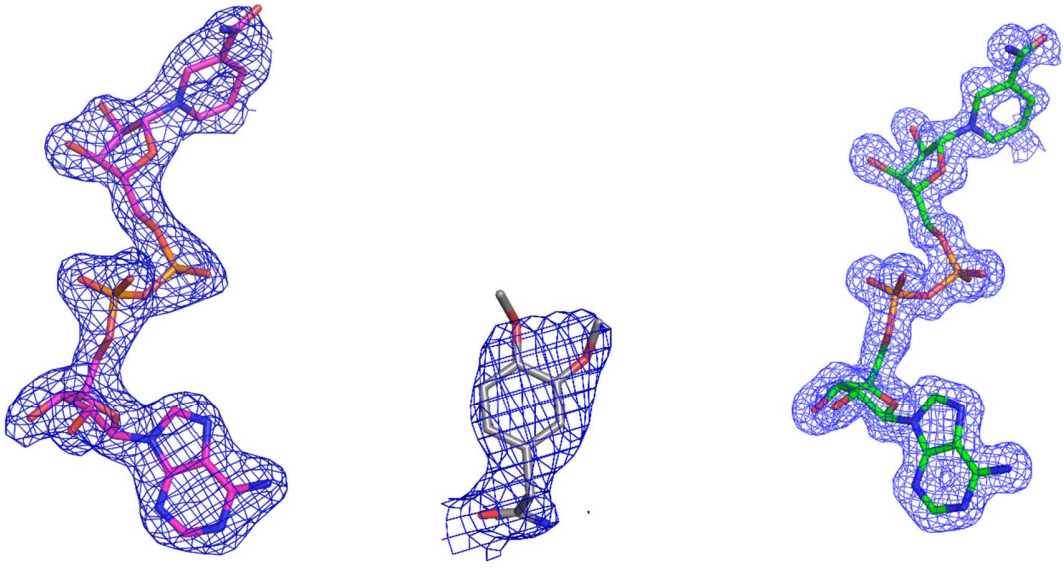
[a] Each crystal was cryoprotected at the end of the crystal treatment protocol by immersing in liquid nitrogen.

[b] Daniel, E., Maksimainen, M. M., Smith, N., Ratas, V., Biterova, E., Murthy, S. N., Rahman, M. T., Kiema, T.-R., Sridhar, S., Cordara, G., Dalwani, S., Venkatesan, R., Prilusky, J., Dym, O., Lehtio, L., Koski, M.K., Ashton, A.W., Sussman, J. L. & Wierenga, R. K. (2021) Acta Cryst. D**77**, 151-163.

Table S2. Medium viscosity effects on reaction rates of the DA1472 variant

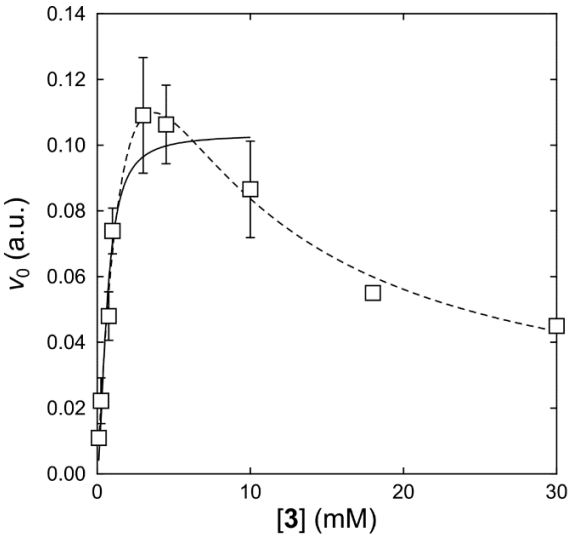
Enzyme	Substrate	η_{rel}	k_{cat} (s^{-1})	k_{cat}/K_M (s^{-1} mM^{-1})	k_{cat} dependency on medium viscosity[a]	k_{cat}/K_M dependency on medium viscosity[a]
DA1472	compound **5**	1	8.0±0.005	80±6		
	compound **5**	1.5	5.6±0.006	43±7	1.2±0.1	1.0±0.2
	compound **5**	2.8	2.5±0.002	27±2		

[a] The slopes are calculated from the linear fits shown in **Fig. S2C**.

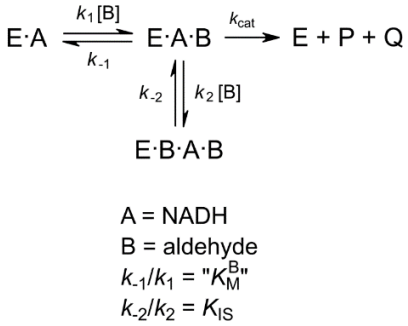


(a) (b) (c)

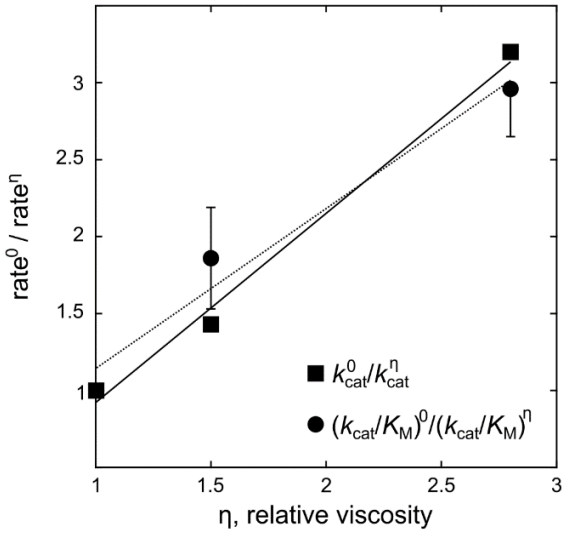
Figure S1. Electron density 2Fo-Fc omit maps, calculated using models in which the specified ligand was omitted from the model. The maps have been contoured at 1 sigma. (a) The DA1472 structure (PDB entry 7QNH) in which NADH of chain B was omitted. (b) The DA1472 structure (PDB entry 7QLS) in which the bound substrate analog (compound **7**, 3,4-dimethoxyphenylacetamide) of chain B was omitted from the model. (c) The D47 structure (PDB entry 7R0P) in which the bound NAD^+ of chain A was omitted.



(*a*)



(*b*)



(*c*)

Figure S2. Kinetic data of the DA1472 variant. (a) Model fitting to steady state data of DA1472 catalyzed reduction of compound **3**. Solid line: Michaelis-Menten model, and dashed line: a model including an additional step of an alternative aldehyde binding mode resulting in a nonproductive dead-end complex. Units of activity is ΔA_{340}/min. The model used is shown in (b). Extracted parameter values (Eq. 3) are given in the main text. (c) Influence of medium viscosity on steady state rate constants k_{cat} (■, solid line) and k_{cat}/K_M (●, dotted line) in the

DA1472 catalyzed reduction of compound **5**. The unit dependencies (**Table S2**) of both parameters suggest product release to be rate limiting for turnover.

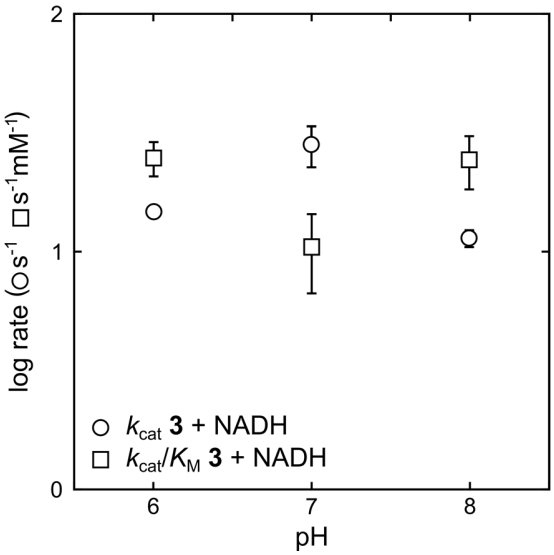


Figure S3. pH dependency of the reduction reaction of the DA1472 variant. The pH dependency of the k_{cat} and k_{cat}/K_M of the DA1472 catalyzed reduction of compound **3** in the presence of 0.4 mM NADH.