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Structure-guided redesign of D-fructose-6-phosphate aldolase from *E. coli*: remarkable activity and selectivity towards acceptor substrates by two-point mutation†

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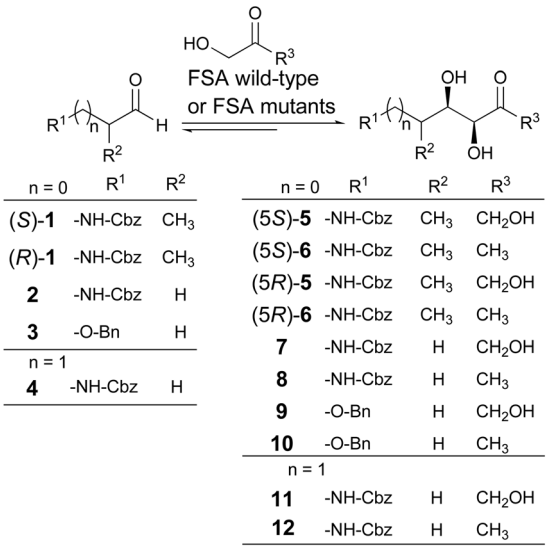
Structure-guided re-design of the acceptor binding site of D-fructose-6-phosphate aldolase from *E. coli* leads to the construction of FSA A129S/A165G double mutant with an activity between 5- to >900-fold higher than that of wild-type towards *N*-Cbz-aminoaldehyde derivatives.

Class I D-fructose-6-phosphate aldolase from *E. coli* (FSA) was recently reported to stereoselectively catalyze (*i.e.* >50 : 1 *syn*/*anti*, 3*S*,4*R* : 3*S*,4*S*) the reversible aldol addition of dihydroxyacetone (DHA), hydroxyacetone (HA) and hydroxybutanone (HB) to a structural variety of acceptor aldehydes.[1,2] Importantly, FSA can also accept glycolaldehyde (GO) as a donor, offering the possibility to perform stereoselective cross-aldol additions of GO in water.[3] Moreover, a single mutation, FSA A129S, improved the reactivity towards DHA by ~17-fold in terms of k_{cat}/K_M, expanding the scope of the preparative competences of the wild-type.[4] Both biocatalysts offer a great advantage over DHAP-dependent aldolases because the direct use of unphosphorylated nucleophiles simplifies enormously the synthesis by avoiding the extra efforts in handling the phosphate group, unless a phosphorylated product in the specific C1 position is desired.

Despite the reported synthetic abilities of FSA and the A129S mutant, limitations arising from the acceptor substrate tolerance have been identified. For instance, no aldol addition of DHA or HA to α-substituted aminoaldehydes, such as (*S*)- and (*R*)-*N*-Cbz-alaninal ((*S*)-**1** and (*R*)-**1**, respectively, Scheme 1), was observed. The respective aldol adducts, (5*S*)-**5** and (5*R*)-**5**, are key intermediates for the expedient synthesis of the pyrrolidine type iminocyclitols, 2,5-imino-1,2,5-trideoxy-D-glucitol and 2,5-imino-1,2,5-trideoxy-D-mannitol (6-deoxy-DMDP), respectively.[5] Alternatively, FruA from rabbit muscle (RAMA), a DHAP-dependent aldolase, rendered 10–46% yield of phosphorylated (5*S*)-**5** and (5*R*)-**5**, respectively.[6] FSA wild-type gave also a disappointing 28–40%‡ yield in the addition of DHA to relatively simple aldehydes such as *O*-benzyloxyacetaldehyde (**3**) and *N*-Cbz-glycinal (**2**).[2] Aldol addition of DHA to **2** furnishes the aldol adduct precursor of 1,4-dideoxy-1,4-imino-D-arabinitol (DAB),[2] which is a potent α-glucosidase inhibitor, and a promising potential therapeutic candidate as an anti-hyperglycemic agent.[7]



n = 0	R^1	R^2
(*S*)-**1**	-NH-Cbz	CH_3
(*R*)-**1**	-NH-Cbz	CH_3
2	-NH-Cbz	H
3	-O-Bn	H
n = 1		
4	-NH-Cbz	H

n = 0	R^1	R^2	R^3
(5*S*)-**5**	-NH-Cbz	CH_3	CH_2OH
(5*S*)-**6**	-NH-Cbz	CH_3	CH_3
(5*R*)-**5**	-NH-Cbz	CH_3	CH_2OH
(5*R*)-**6**	-NH-Cbz	CH_3	CH_3
7	-NH-Cbz	H	CH_2OH
8	-NH-Cbz	H	CH_3
9	-O-Bn	H	CH_2OH
10	-O-Bn	H	CH_3
n = 1			
11	-NH-Cbz	H	CH_2OH
12	-NH-Cbz	H	CH_3

Scheme 1 Aldol addition of DHA and HA to selected aldehydes catalysed by FSA (wt = wild-type).

In this work, we endeavoured to re-design rationally the acceptor-binding site of the FSA wild-type and the FSA A129S mutant by structure-guided site directed mutagenesis. Our goal was to modify the acceptor aldehyde binding pocket of both FSA enzymes to improve their catalytic performance towards *N*-Cbz-aminoaldehydes without altering their high stereoselectivity,[2,4] thus enlarging the number of tolerated substrates and expanding their general utility in asymmetric carbon–carbon bond formation.

The crystallographic structure of FSA and the minimized modelled hemiaminal of D-fructose-6-phosphate (F6P) and K85 revealed two putative residues that might be related with

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† Electronic supplementary information (ESI) available: General procedures, site-directed mutagenesis, electrospray ionization mass spectrometry of the FSA mutants, specific aldolase activity of the FSA wild-type and the mutants, preliminary screening of the aldol addition reactions catalysed by FSA wild-type and mutants, analytical methods, determination of initial velocities and steady state kinetic parameters, preparation of aldol adducts (5*S*)-**5**, (5*S*)-**6**, (5*R*)-**5**, (5*R*)-**6**, and **7** and conversion into the corresponding iminocyclitols and copies of their NMR spectra including aldol adduct **11**. See DOI: 10.1039/c1cc11069a

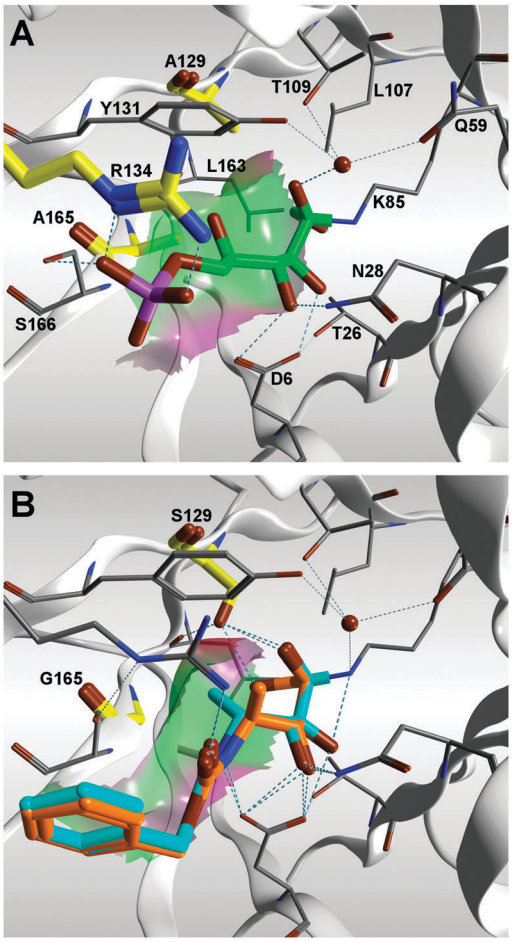

Fig. 1 (A) Modelled F6P-derived hemiaminal (green) in the active centre of FSA wild-type. Residues considered for mutation are shown in yellow. (B) Modelled hemiaminal complexes of (5*R*)-**5** (cyan) and (5*S*)-**5** (orange) in the active centre of the FSA A129S/A165G mutant. Residues mutated are shown in yellow. An essential water molecule is shown as a red ball. The surface of the protein close to residue 165 shows the increased available space in the double mutant relative to the wild-type protein, which allows allocation of the C-α methyl group of **5** without steric clashes with the protein.

the acceptor substrate-binding site (see Fig. 1A). Residue R134 appears to be involved in accommodating phosphorylated acceptors such as the D-glyceraldehyde-3-phosphate, by analogy to that equivalent of transaldolase B from *E. coli*,[8] and thus may interfere with large hydrophobic non-phosphorylated aldehydes such as the ones selected in this study. Therefore, substitution of alanine for arginine was envisaged as a way to favour the accommodation of bulky substrates. The second residue was the A165, which is oriented towards the covalent complex between DHA/F6P and the conserved Lys residue. Substitution of glycine for alanine may allow more space and flexibility to allocate the aldehydes, especially those C-α substituted. Therefore, FSA R134A and FSA A165G mutants were constructed and investigated as biocatalysts for the aldol addition of DHA and HA to aldehydes **1–4** (Scheme 1).

The results (Table 1 and ESI†) revealed that the FSA A165G provided good to excellent conversions to aldol adducts for the selected acceptor aldehydes, at initial velocities from 4 to 175-fold higher than those of FSA wild-type either with DHA or HA donors. Furthermore, both enantiomers of C-α substituted Cbz-alaninal, which were not substrates for the FSA wild-type, were tolerated as acceptors.

The FSA A129S mutant gave also improved conversions in the aldol addition of DHA albeit it showed similar acceptor selectivity as compared with FSA wild-type.[4] FSA R134A gave comparable or lower conversions than that with FSA wild-type for the selected aldehydes (see ESI†). Then, we reasoned that the higher affinity of FSA A129S for DHA combined with the wider aldehyde tolerance of FSA A165G might benefit from a synergistic effect of these two mutations. Indeed, the FSA A129S/A165G double mutant resulted to be the best biocatalyst for the aldol addition of DHA and HA to the selected aldehydes. Unprecedented excellent results were obtained for the aldol addition reactions of DHA to aldehydes (*S*)-**1** and (*R*)-**1**, whereas no reaction was detected using FSA wild-type, FSA R134A or FSA A129S. Models of the covalent complexes of adducts (5*S*)-**5** and (5*R*)-**5** in the active centre of the A129S/A165G double mutant showed, as expected, that the A165G mutation generates the required space to allocate the C-α methyl group without clashing with the

Table 1 FSA-catalyzed aldol addition of DHA or HA to aldehydes **1–4**. Initial reaction rates (ν_o) and percentage of aldol adduct formation

Acceptor aldehyde	Donor	ν_o[a]/μmol min^{-1} mg^{-1}				Aldol adduct,[b] % (protein used/mg)			
		FSA wt	FSA A129S	FSA A165G	FSA A129S/A165G	FSA wt	FSA A129S	FSA A165G	FSA A129S/A165G
(*S*)-**1**	DHA	nd[c]	nd[c]	0.10 ± 0.03	0.6 ± 0.1	nd[c](1.0)	nd[c](1.0)	51(0.5)	78(0.5)
	HA	0.014 ± 0.001	nd[c]	3.8 ± 0.2	13 ± 2	31(0.5)	21(0.5)	85(0.05)	92(0.05)
(*R*)-**1**	DHA	nd[c]	nd[c]	0.33 ± 0.01	0.5 ± 0.1	nd[c](1.0)	nd[c](1.0)	73(0.5)	95(0.5)
	HA	nd[c]	nd[c]	4.2 ± 0.3	8.3 ± 0.4	7(1.0)	7(1.0)	84(0.05)	95(0.05)
2	DHA	0.02 ± 0.01	0.28 ± 0.04	3.5 ± 0.4	15 ± 1	25(1.0)	56(1.0)	100(0.1)	100(0.05)
	HA	1.3 ± 0.2	1.5 ± 0.1	20 ± 2	49 ± 3	97(0.1)	100(0.1)	100(0.1)	100(0.01)
3	DHA	0.02 ± 0.01	0.13 ± 0.03	0.48 ± 0.03	2.1 ± 0.2	8(0.85)	60(0.85)	81(0.9)	100(0.85)
	HA	0.9 ± 0.1	0.77 ± 0.02	3.2 ± 0.3	10 ± 1	92(0.1)	93(0.1)	95(0.05)	100(0.05)
4	DHA	0.26 ± 0.03	0.9 ± 0.1	1.2 ± 0.2	9.0 ± 0.2	74(0.85)	83(0.3)	91(0.3)	99(0.1)
	HA	2.9 ± 0.5	2.4 ± 0.3	12.0 ± 1.0	38 ± 3	86(0.1)	90(0.1)	90(0.1)	96(0.05)

[a] [DHA] or [HA] (100 mM) and acceptor aldehydes (80 mM) total volume 300 μL. Different amounts of enzyme were used for obtaining a linear dependence of the concentration *versus* time between 0% and 10% of conversion. Values are the mean of four independent determinations ± standard error. [b] Product formation measured by HPLC after 24 h using an external standard method. Values are the mean of two independent determinations (see ESI†); percentage calculated with respect to the limiting acceptor aldehyde substrate. [c] nd: not detected (*i.e.* ≤0.01 μmol min^{-1} mg^{-1}) or no peak of the aldol adduct detected by HPLC (detection limit: 0.2–0.5 nmol).

Table 2 Apparent V_{max}^{app} and K_m^{app} values of DHA and **4** for their aldol addition reaction

FSA catalysts	Substrate	V_{max}^{app}/μmol min^{-1} mg^{-1}	K_m^{app}/mM	$10^3 \times V_{max}^{app}/K_m^{app}$/min^{-1} mg^{-1}
Wild-type	**4**[a]	0.8 ± 0.1	169 ± 19[b]	17
A129S	**4**	1.9 ± 0.2	139 ± 24[b]	47
A165G	**4**	1.9 ± 0.2	122 ± 15[c]	50
A129S/A165G	**4**	12 ± 1	24 ± 5[c]	1733
Wild-type	DHA	0.46 ± 0.02	58 ± 6	27
A129S	DHA	2.2 ± 0.1	7 ± 2	1000
A165G	DHA	2.2 ± 0.2	22 ± 6	333
A129S/A165G	DHA	15 ± 2	32 ± 13	1533

[a] Concentration of **4** could not be higher than 200 mM due to the limited solubility in buffer/DMF 4 : 1 mixtures. [b] Max [**4**] = 200 mM. [c] Max [**4**] = 140 mM.

protein residues (Fig. 1B). Furthermore, the models also suggest that the mutated S129 residue can establish hydrogen bonds with two of the oxygen atoms of the DHA moiety. This could increase the nucleophilicity of the reactive carbon of DHA, which would explain the increased activity observed for enzymes that contain such mutation. Remarkably good results were also obtained with the HA donor, which could not be anticipated since FSA wild-type and the A129S mutant showed similar velocities for this nucleophile. Furthermore, the initial reaction velocities (ν_o) supported evidence of the synergistic effect of the double mutation on FSA. As shown in Table 1, the improved activity of the double mutation was 4- to 10-fold higher than that of individual mutations for DHA and 3-fold higher than those for HA.

Importantly, the results indicate that the stereochemical outcome of the FSA mutants towards the selected substrates was identical to that of FSA wild-type, therefore the mutations did not change the preferential orientation of the selected donor and acceptor substrates at the FSA active site (see ESI†). To gain insight into the catalytic properties of FSA wild-type and mutants, the apparent steady state kinetic parameters for the nucleophile DHA and acceptor **4** were determined (Table 2).

As expected, in terms of V_{max}^{app}/K_m^{app} the double mutant functions with aldehyde **4** ~100-fold better than the FSA wild-type, mainly due to a ~15-fold increase in V_{max}^{app} and a ~7-fold decrease in K_m^{app}. Moreover, as judged by the V_{max}^{app}/K_m^{app}, the FSA A129S/A165G functions with the nucleophile, DHA, 50-fold better than the FSA wild-type. It is noteworthy that FSA A129S has still better K_m^{app} for DHA, in good agreement with previous observations,[4] but lower V_{max}^{app} than the double mutant. The higher V_{max}^{app} of FSA A129S/A165G compensates the K_m^{app} value turning out to be the best mutant for DHA. The V_{max}^{app}/K_m^{app} values for aldehyde **4** imply that the double mutant is much more efficient in transforming this substrate than the FSA wild-type. The results obtained may be extrapolated to the other selected substrates and are consistent with the fact that the best catalyst for the aldol addition reactions of DHA to aldehydes (*S*)-**1**, (*R*)-**1**, **2**, and **3** is the FSA A129S/A165G double mutant.

To sum up, the FSA A129S/A165G double mutant constitutes a promising biocatalyst for *syn* configured aldol reactions of DHA and HA with wider acceptor aldehyde selectivity as compared with FSA wild-type. Examples of isolated yields of aldol adducts improved significantly as compared with FSA wild-type and RAMA: (5*S*)-**5** (from 10 to 83%), (5*R*)-**5** (from 46 to 72%) and **7** (from 40 to 82%) (see ESI†). Furthermore, gaining insight into identifying the critical residues for the acceptor-binding site of FSA led to such improved catalytic efficiency. The structure-guided approach based on site-directed mutagenesis can be, in this case, a potent tool for further re-designs of FSA and related aldolases to suit any future specific targets. Work towards this direction is currently in progress in our lab.

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‡ Efforts to improve the yields by optimizing the reaction conditions resulted unfruitful.

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