Binding energy and specificity in the catalytic mechanism of yeast aldose reductases

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Derivatives of D-xylose and D-glucose, in which the hydroxy groups at C-5, and C-5 and C-6 were replaced by fluorine, hydrogen and azide, were synthesized and used as substrates of the NAD(P)H-dependent aldehyde reduction catalysed by aldose reductases isolated from the yeasts *Candida tenuis*, *C. intermedia* and *Cryptococcus flavus*. Steady-state kinetic analysis showed that, in comparison with the parent aldoses, the derivatives were reduced with up to 3000-fold increased catalytic efficiencies (k_{cat}/K_m), reflecting apparent substrate binding constants (K_m) decreased to as little as 1/250 and, for D-glucose derivatives, up to 5.5-fold increased maximum initial rates (k_{cat}). The effects on K_m mirror the relative proportion of free aldehyde that is available in aqueous solution for binding to the binary complex enzyme–NAD(P)H. The effects on k_{cat} reflect non-productive binding of the pyranose ring of sugars; this occurs preferentially with the NADPH-dependent enzymes. No transition-state stabilization energy seems to be derived from hydrogen-bonding interactions between enzyme–NAD(P)H and positions C-5 and C-6 of the aldose. In contrast, unfavourable interactions with the C-6 group are used together with non-productive binding to bring about specificity (6–10 kJ/mol) in a series of D-aldoses and to prevent the reaction with poor substrates such as D-glucose. Azide introduced at C-5 or C-6 destabilizes the transition state of reduction of the corresponding hydrogen-substituted aldoses by approx. 4–9 kJ/mol. The total transition state stabilization energy derived from hydrogen bonds between hydroxy groups of the substrate and enzyme–NAD(P)H is similar for all yeast aldose reductases (yALRs), at approx. 12–17 kJ/mol. Three out of four yALRs manage on only hydrophobic enzyme–substrate interactions to achieve optimal k_{cat}, whereas the NAD(P)H-dependent enzyme from *C. intermedia* requires additional, probably hydrogen-bonding, interactions with the substrate for efficient turnover.

Key words: aldo/keto reductases, aldose derivatives, substrate analogues.

INTRODUCTION

The catabolic pathway of D-xylose in yeast starts with the NAD(P)H-dependent reduction of D-xylose, which is catalysed by aldose reductase and yields xylitol. The yeast aldose reductases (yALRs) are functional monomers or dimers composed of approx. 36 kDa protein subunits [1]. They are members of the aldo/keto reductase superfamily [1,2]. However, recent sequence comparison [1] has revealed some additional features of yALRs that are characteristic of short-chain dehydrogenases/reductases rather than ALRs. However, it is not yet clear whether these findings bear significance concerning the function of yALRs in comparison with that of mammalian aldose reductases (mALRs). According to their coenzyme specificity, yALRs are separated into a NADPH-specific group (see [3,4]) and another group in which enzymes show comparable specificity constants with NADPH and NADH (see [5,6]). yALRs with dual coenzyme specificity are probably essential for the utilization of D-xylose by yeast under oxygen-limited growth conditions (reviewed in [7]).

In spite of overall structural similarities [1], mALR and yALR show different kinetic properties. mALR binds NADPH very tightly and thereby creates an extremely reactive binary enzyme–nucleotide complex, capable of reducing a great number of structurally diverse aldehydes with similar catalytic efficiencies (k_{cat}/K_m) [8]. At substrate saturation, the steady-state reaction rate is determined entirely by the isomerization of the enzyme–$NADP^+$ complex, which precedes the dissociation of the oxidized coenzyme [9,10]. As a consequence, k_{cat} values of mALR are almost independent of the structure of non-reacting portions of the aldehyde substrate. We have shown recently [11] that in marked contrast with mALR, yALR from *Candida tenuis* binds NAD(P)H weakly and uses binding energy derived from hydrogen-bonding interactions, particularly with the C-2 (*R*) hydroxy group of aldose substrates, to decrease the activation free energy for the rate-determining step. For example, k_{cat} of yALR with D-galactose was 15-fold that seen with 2-fluoro-2-deoxy-D-galactose, in which those hydrogen bonds with the yALR–NADH complex were removed that involve the C-2 hydroxy group as the donor. In addition, hydrophobic bonding interactions with C-3 to C-6 of straight-chain aldehydes were found to bring about an approx. 200-fold increase in k_{cat}/K_m, reflecting mainly the apparent tighter substrate binding with increasing chain length of the aldehyde. Once correction has been made for the percentage of free aldehyde present in aqueous solution, k_{cat}/K_m values of yALR seem to be highest [approx. $(1–2)\times10^6\ M^{-1}\cdot s^{-1}$] for D-xylose and L-arabinose [11]. In contrast, with a value for k_{cat}/K_m of approx. $(0.4–2)\times10^5\ M^{-1}\cdot s^{-1}$, D-glucose is the poorest substrate of yALR in a series of D-aldoses [11]. The mechanism by which yALR differs up to 50-fold between D-xylose and D-glucose is not understood. However, it could be important to prevent the reaction with D-glucose during the growth of xylose-metabolizing yeasts on natural substrates, which typically will contain both D-xylose and D-glucose as the major carbon sources.

Abbreviations used: ALR, aldose reductase; CHCA, cyclohexanecarboxaldehyde; mALR, mammalian aldose reductase; yALR, yeast aldose reductase.

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The aim of this work was to investigate the determinants of substrate specificity in NADPH-dependent and NAD(P)H-dependent yALR. To this end, derivatives of D-xylose and D-glucose were synthesized in which the hydroxy groups at C-5, and C-5 and C-6 were replaced by fluorine, hydrogen or azide. They are introduced here as a new class of 'tight-binding' polyhydroxylated aldehyde substrates of yALR. By using a steady-state kinetic analysis of the NAD(P)H-dependent reduction of these derivatives, compared with that of the parent aldoses, it was possible to show that, unexpectedly for a carbohydrate-binding protein, yALR does not donate hydrogen bonds to hydroxy groups at positions C-5 and C-6 of the aldose substrate so as to bring about specificity. Non-productive binding is the mechanism used by NADPH-dependent yALR to distinguish between different D-aldoses, resulting in a decrease in k_{cat} for poor substrates such as D-glucose in comparison with D-xylose, for example.

EXPERIMENTAL

yALRs

Yeast strains labelled HA and HB were obtained from a yeast culture collection maintained at the Institute of Applied Microbiology (BOKU, Vienna, Austria). The NAD(P)H-dependent ALR from *C. tenuis* CBS 4435 was isolated as decribed previously [6]. The NADPH-dependent and the NAD(P)H-dependent ALR from *Candida intermedia* HA 409 were isolated by procedures identical with that reported for the ALR from *C. tenuis*, with the exception that the dye Procion Red HE3B (colour index Reactive Red 120; ICI Chemicals) was used in the first step as pseudo-affinity ligand coupled to Sepharose CL-4B (details available from the authors). The NADPH-dependent ALR from *Cryptococcus flavus* HB 402 was purified by a two-step protocol that included (1) hydrophobic interaction chromatography on Phenyl-Sepharose 6 Fast Flow (high sub), using the crude cell extract dissolved in 25%-satd. $(NH_4)_2SO_4$ and eluting with a linear gradient of decreasing saturation with $(NH_4)_2SO_4$; (2) pseudo-affinity chromatography of the desalted ALR preparation on a column of the dye H8B (colour index Reactive Red 31; ICI Chemicals) coupled to Sepharose CL-4B, with NaCl for elution. The individual enzyme preparations seemed to be pure, in that each ALR migrated as a single band in SDS/PAGE, non-denaturing PAGE and isoelectric focusing (results not shown). The molecular mass of each ALR was determined from SDS/PAGE (PhastGel Homogeneous 20; Amersham Pharmacia) and matrix-assisted laser ionization desorption MS. An average molecular mass of 36 ± 2 kDa was obtained, and there was only marginal variation between individual ALRs, although previous results with PhastGel Gradient 8-25 had indicated a slightly higher molecular mass (43 kDa) for the ALR from *C. tenuis* [6].

Derivatives of D-xylose and D-glucose

Reported protocols were used for the synthesis of 5-deoxy-D-xylofuranose (**1**) [12], 5-fluoro-5-deoxy-D-xylofuranose (**2**) [13] and 5-azido-5-deoxy-D-xylofuranose (**3**) [14].

The general strategy for the preparation of substrates **4**–**9** [(5,6-dideoxy-5,6-difluoro-D-glucofuranose (**4**); 6-azido-5,6-dideoxy-5-fluoro-D-glucofuranose (**5**); 5,6-dideoxy-5-fluoro-D-glucofuranose (**6**); 5,6-dideoxy-6-fluoro-D-xylohexofuranose (**7**); 5,6-dideoxy-D-xylohexofuranose (**8**) and 6-azido-5,6-dideoxy-D-xylohexofuranose (**9**)] was as follows. Modification of C-5 with overall retention of configuration was achieved in accordance with published procedures [15–17] employing 1,2-*O*-isopropylidene-α-D-glucofuranurono-6,3-lactone as a readily available starting material. Reduction of the modified lactone gave the corresponding diol. Attempts to regiospecifically sulphonate the primary hydroxy group at C-6 failed owing to intramolecular 6,3-anhydro ring formation. To avoid this problem, OH-6 was *O*-sulphonylated with trifluoromethanesulphonic anhydride after protection of OH-3. Subsequently, azide, fluoride or iodide was successfully introduced to give high yields of fully protected 5,6-dimodified glucofuranoses. Catalytic reduction of the deoxyiodo sugars led to the corresponding deoxy compounds. Conventional acidic deprotection furnished anomeric mixtures of the free 5,6-dimodified D-glucofuranoses (**4**)–(**9**). Selected results are as follows: (**4**), [α]-5.3 $c = 1.4$ (CH_3OH); ^{13}C NMR (^2H_2O): δ 102.0 (C-1β), 96.2 (C-1α), 89.0, 88.8 ($J_{5,F}$ 161.9/162.1 Hz, C-5α/C-5β), 82.9, 79.7 ($J_{4,F}$ 28.1/26.4 Hz, C-4α/C-4β) 80.3, 75.8 (C-2α/C-2β), 74.8, 74.5 ($J_{3,F}$ 3.3/2.1 Hz, C-3α/C-3β), 17.5, 16.9 ($J_{6,5}$ 21.5/21.1 Hz, C-6α/C-6β); (**6**), [α]-6.7 $c = 3.25$ (CH_3OH); ^{13}C NMR (^2H_2O): δ 102.4 (C-1β), 96.8 (C-1α), 89.7, 89.3 ($J_{5,F\text{-}5}$ 170.2/169.2 Hz, $J_{5,F\text{-}6}$ 18.2/17.7 Hz, C-5α/C-5β), 83.6, 82.9 ($J_{6,F\text{-}6}$ 167.6 Hz, $J_{6,F\text{-}5}$ 19.3/19.2 Hz, C-6α/C-6β), 80.1, 75.6 (C-2α/C-2β), 77.9, 75.1 ($J_{4,F\text{-}5}$ 29.5/28.6 Hz, $J_{4,F\text{-}6}$ 7.2/6.9 Hz, C-4α/C-4β), 74.8, 74.4 ($J_{3,F\text{-}5}$ 1.6 Hz, C-3α/C-3β).

Initial velocity studies and determination of kinetic parameters

Measurements of the initial rate of aldehyde reduction were performed with a Beckman DU-650 spectrophotometer at 25 °C with NADH or NADPH as coenzyme. The oxidation of coenzyme on aldehyde reduction was monitored at 340 nm (1–5 min, rate 0.05–0.1 ΔA/min). All rates were corrected for the appropriate blank readings, lacking either the substrate or the enzyme. The standard reaction mixture had a total volume of 1 ml. It contained approx. 15–150 nM yALR and a constant saturating concentration of 220 μM NAD(P)H (approx. 10–20-fold $K_m^{NAD(P)H}$), dissolved in 50 mM potassium phosphate buffer, pH 7.0. The aldehyde substrate was varied, typically in five to seven different concentration points, over a concentration range covering approx. 0.1–10-fold the $K^{RO}{}_m$ for each aldehyde. Unless mentioned otherwise, apparent saturation was achieved with all substrates, and at least two concentration points in the region of $K^{RO}{}_m$ were measured. Substrates poorly soluble in water, such as oenanthaldehyde or cyclohexanecarboxaldehyde (CHCA), were dissolved in 99% (v/v) ethanol. Reaction mixtures for kinetic measurements were prepared by diluting the aldehyde into phosphate buffer to give a final ethanol concentration of 2% (v/v). It was proved that this amount of ethanol had no effect on the kinetics of D-xylose reduction.

All kinetic parameters were calculated by fitting the Michaelis–Menten function [eqn. (1)] directly to the data (SigmaPlot, version 5) by using an unweighted non-linear least-squares analysis:

$$V(RO) = k_{cat} \cdot [E] \cdot [RO]/(K^{RO}{}_m + [RO]) \quad (1)$$

When substrate inhibition occurred, eqn. (2) was used:

$$V(RO) = k_{cat} \cdot [E] \cdot [RO]/(K^{RO}{}_m + [RO] + [RO]^2/K^S{}_i) \quad (2)$$

where V(RO) is the initial velocity, k_{cat} is the catalytic constant, [E] is the total concentration of yALR (taking a molecular mass of 36 kDa for calculations), [RO] is the concentration of varied aldehyde, $K^{RO}{}_m$ is the apparent Michaelis constant for RO and $K^S{}_i$ is the substrate inhibition constant for RO.

Other measurements

Optical rotations were measured on a Jasco Digital Polarimeter with a path length of 10 cm. NMR measurements were performed at 25 °C with a Bruker MSL 300 instrument. ^{13}C NMR spectra

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were recorded at 50.29 MHz. ^{1}H NMR spectra were obtained at 300.13 MHz. Chemical shifts are listed in δ with residual, not deuterated, solvent as the internal standard.

Other materials

NAD^+-dependent formate dehydrogenase was from ASA Spezialenzyme GmbH (Braunschweig, Germany). $NADP^+$-dependent formate dehydrogenase was obtained from Immunotech (Moscow, Russia). All other reagents and chemicals were of the highest purity available.

RESULTS

Interaction of yALR with D-xylose derivatives

The NAD(P)H-dependent reduction of D-xylose and derivatives of D-xylose, in which the C-5 hydroxy group was replaced by hydrogen (**1**), fluorine (**2**) or azide (**3**), was measured by using yALR as the enzyme catalyst. Four individual ALRs from three different yeast strains were purified to homogeneity and compared in these experiments. The enzyme selection reflects completely the multiplicity of ALRs in xylose-metabolizing yeasts. It was made to provide either a general picture or otherwise a differential view on enzyme–substrate interactions in yALR. In *Cr. flavus* (P. Mayr and B. Nidetzky, unpublished work) and *C. tenuis* [6], one single ALR enzyme is found. That in *Cr. flavus* is strictly specific for NADPH ($k_{cat}/K_m^{NADPH} = 8.08 \times 10^5$ $M^{-1} \cdot s^{-1}$), whereas that in *C. tenuis* has a dual coenzyme specificity ($k_{cat}/K_m^{NADH} = 7.13 \times 10^5$ $M^{-1} \cdot s^{-1}$ [6]). In contrast, *C. intermedia* produces two different ALR enzymes. One is strictly specific for NADPH ($k_{cat}/K_m^{NADPH} = 4.00 \times 10^5$ $M^{-1} \cdot s^{-1}$), whereas the other shows similar specificity constants for NADPH ($k_{cat}/K_m^{NADPH} = 5.48 \times 10^5$ $M^{-1} \cdot s^{-1}$) and NADH ($k_{cat}/K_m^{NADH} = 2.97 \times 10^5$ $M^{-1} \cdot s^{-1}$). All four yALRs showed remarkably similar catalytic efficiencies with D-xylose as aldehyde substrate (k_{cat}/K_m^{xylose} in Tables 1 and 2).

The results of a steady-state kinetic analysis of the measured initial velocities are summarized in Tables 1 and 2. Replacement of the C-5 hydroxy group in the derivatives (**1**)–(**3**) had a strong influence on the kinetic parameters of each yALR. A 31–145-fold increase in $k_{cat}/K^{RO}{}_m$, relative to the corresponding $k_{cat}/K^{RO}{}_m$ value for D-xylose, was observed with compounds (**1**) and (**2**) (Tables 1 and 2). From $\Delta\Delta G = -RT\ln(k_{cat}/K^{RO}{}_m)_{derivative}/(k_{cat}/K^{RO}{}_m)_{D\text{-}xylose}$ the effects on $k_{cat}/K^{RO}{}_m$ translate into apparent extra binding energies of 8.5–12.3 kJ/mol used for transition state stabilization. With compound (**3**) the increase in $k_{cat}/K^{RO}{}_m$, relative to the corresponding $k_{cat}/K^{RO}{}_m$ value with D-xylose, was in the range 1.6-fold to approx. 8.6-fold. The kinetic parameters for (**3**), that is k_{cat} and $K^{RO}{}_m$, could not be determined separately in all instances (Tables 1 and 2) because $K^{RO}{}_m$ was higher than 20 mM and, given the limited availability of (**3**), saturation with substrate could not be achieved in the experiment. Therefore $k_{cat}/K^{RO}{}_m$ was determined from the part of the Michaelis–Menten curve where the initial velocity is linearly dependent on the substrate concentration. The effects on $k_{cat}/K^{RO}{}_m$ seen with compounds (**1**)–(**3**) were due to (1) large decreases in $K^{RO}{}_m$, and (2) weaker but significant increases in k_{cat} (approx. 1.3–1.8-fold). In the ^{1}H-NMR experiment at 25 °C, the acyclic aldehyde forms of (**1**), (**2**) and (**3**) were detectable in small amounts (1–2 %) in ^2H_2O solution, p^{2}H 7.0. The proportion of free aldehyde species in aqueous solutions of D-xylose is 0.02 % [18] (Scheme 1a). If we assume a proportion of free aldehyde of 1.5 % for the D-xylose derivatives, and furthermore that yALR binds its substrates in the free aldehyde form, as does mALR [19], a decrease in $K^{RO}{}_m$

Table 1 Kinetic parameters for NADPH-dependent reduction of xylose derivatives by yALR

The concentration of NADPH was 220 μM and saturating. Reaction conditions: 50 mM potassium phosphate buffer, pH 7.0, 25 °C. For $K^{RO}{}_m$ and $k_{cat}/K^{RO}{}_m$ the ratio is given (in parenthesis) of the observed value to the value expected on comparison of the percentage of free aldehyde form in aqueous solution of xylose (0.02%) or derivatives (1.5%). $\Delta\Delta G$ was calculated from $\Delta\Delta G = -RT\ln[(k_{cat}/K^{RO}{}_m)_{deriv}/(k_{cat}/K^{RO}{}_m)_{xylose}]$; $\Delta\Delta G_{predicted} - \Delta\Delta G_{observed}$ is shown in parenthesis; $\Delta\Delta G_{predicted}$ was $-RT\ln[1.5/0.02]$. In this and subsequent tables the compound number is given in bold in the substrate column. Abbreviation: n.d., not determined.

	Cryptococcus flavus				*Candida intermedia* I			
Substrate	k_{cat} (s^{-1})	$K^{RO}{}_m$ (mM)	$k_{cat}/K^{RO}{}_m$ ($M^{-1} \cdot s^{-1}$)	$-\Delta\Delta G$ (kJ/mol)	k_{cat} (s^{-1})	$K^{RO}{}_m$ (mM)	$k_{cat}/K^{RO}{}_m$ ($M^{-1} \cdot s^{-1}$)	$-\Delta\Delta G$ (kJ/mol)
Xylose	8.8 ± 0.5	27 ± 2	326	–	14.6 ± 0.5	82 ± 8	178	–
5-Deoxy (**1**)	12.5 ± 0.6	0.7 ± 0.0 (1.9)	17 857 (0.7)	9.9 (−0.8)	18.1 ± 0.6	1.0 ± 0.1 (0.9)	18 100 (1.4)	11.4 (0.7)
5-Fluoro (**2**)	15.6 ± 0.6	1.0 ± 0.2 (2.8)	15 600 (0.6)	9.6 (−1.1)	20.0 ± 3.1	1.6 ± 0.7 (1.5)	12 500 (0.9)	10.5 (−0.2)
5-Azido (**3**)	11.2 ± 1.2	4.0 ± 0.9 (11)	2 800 (0.1)	5.3 (−5.4)	n.d.	n.d.	1 120 (0.08)	4.5 (−6.2)

Table 2 Kinetic parameters for NADH-dependent reduction of xylose derivatives by yALR

The concentration of NADH was 220 μM and saturating. Reaction conditions: 50 mM potassium phosphate buffer, pH 7.0, 25 °C. For $K^{RO}{}_m$ and $k_{cat}/K^{RO}{}_m$ the ratio is given (in parenthesis) of the observed value to the value expected on comparison of the percentage of free aldehyde form in aqueous solution of xylose (0.02%) or derivatives (1.5%). $\Delta\Delta G$ was calculated from $\Delta\Delta G = -RT\ln[(k_{cat}/K^{RO}{}_m)_{deriv}/(k_{cat}/K^{RO}{}_m)_{xylose}]$; $\Delta\Delta G_{predicted} - \Delta\Delta G_{observed}$ is shown in parenthesis; $\Delta\Delta G_{predicted}$ was $-RT\ln[1.5/0.02]$. Abbreviation: n.d., not determined.

	Candida tenuis				*Candida intermedia* II			
Substrate	k_{cat} (s^{-1})	$K^{RO}{}_m$ (mM)	$k_{cat}/K^{RO}{}_m$ ($M^{-1} \cdot s^{-1}$)	$-\Delta\Delta G$ (kJ/mol)	k_{cat} (s^{-1})	$K^{RO}{}_m$ (mM)	$k_{cat}/K^{RO}{}_m$ ($M^{-1} \cdot s^{-1}$)	$-\Delta\Delta G$ (kJ/mol)
Xylose	14 ± 1	64 ± 6	219	–	10.5 ± 0.6	50 ± 5	210	–
5-Deoxy (**1**)	18 ± 1	0.9 ± 0.3 (1.0)	20 000 (1.2)	11.2 (0.5)	13.5 ± 0.6	1.3 ± 0.3 (2.0)	10 384 (0.7)	9.7 (−1.0)
5-Fluoro (**2**)	19 ± 1	0.6 ± 0.4 (0.7)	31 667 (1.9)	12.3 (1.6)	15.5 ± 1.9	2.4 ± 0.8 (3.6)	6 458 (0.4)	8.5 (−2.2)
5-Azido (**3**)	n.d.	n.d.	1 690 (0.1)	5.1 (−5.6)	n.d.	n.d	346 (0.02)	1.2 (−9.5)

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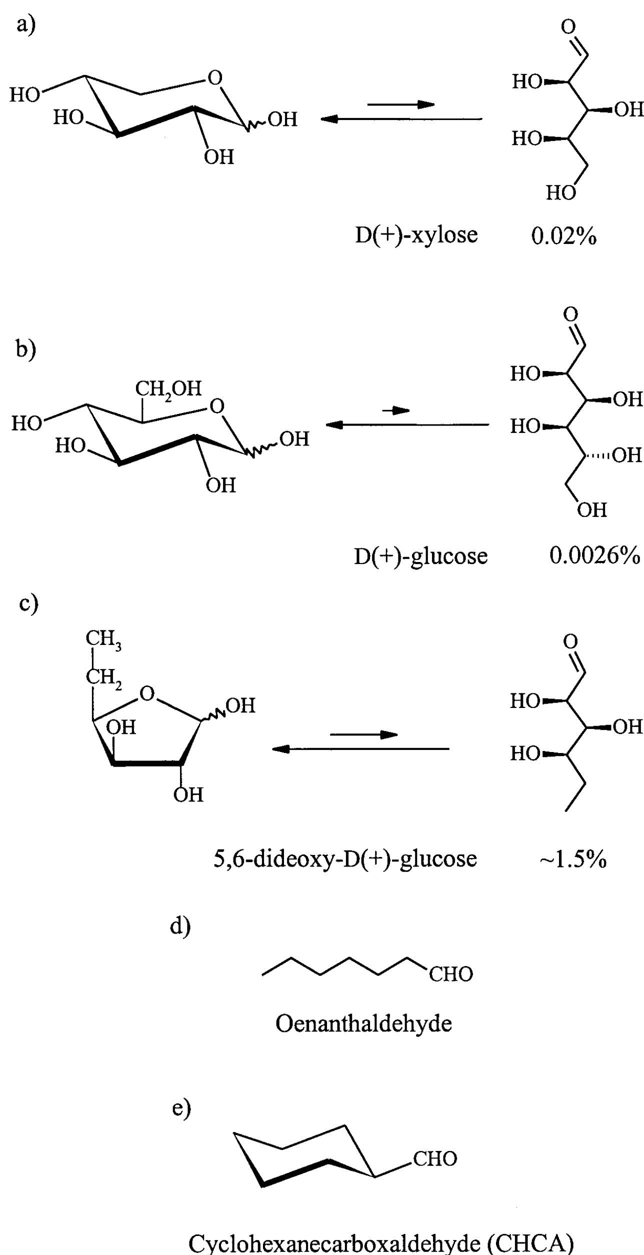

Scheme 1 Structures of ALR substrates, indicating the proportions of free aldehyde present in aqueous solution

to 1/75, relative to K^{RO}_m for D-xylose, would be expected for (**1**)–(**3**). The predicted effect on K^{RO}_m is in good agreement with the experimental findings for (**1**) and (**2**) but not (**3**) (see below). The differences in free energy for the reduction of (**1**) and (**2**) by yALRs were calculated by using the values for k_{cat}/K^{RO}_m in Tables 1 and 2. They were usually within a factor of approx. RT. Therefore yALRs show little, if any, difference in specificity concerning the replacement of OH by H or F at C-5. With compound (**3**), values for k_{cat}/K^{RO}_m were 1/10 to 1/46 times those predicted by simply comparing the proportions of free aldehyde forms in aqueous solutions of D-xylose and (**3**). By using the catalytic efficiencies in Tables 1 and 2, a difference in free energy of between 4.2 and 8.4 kJ/mol is calculated for the yALR-catalysed reduction of compound (**3**) on the one hand and compounds (**1**) or (**2**) on the other.

NAD(P)H-dependent reduction of D-glucose derivatives

On comparing the kinetic parameters for yALR-catalysed reduction of D-glucose and derivatives (**4**)–(**9**) in Tables 3 and 4, two general effects are immediately clear. The replacement of the C-5 and C-6 hydroxy groups leads to (1) a 3.7–5.5-fold increase in k_{cat} and (2) a decrease in K^{RO}_m to as little as 1/250, both relative to the corresponding kinetic parameters for D-glucose. In case of NAD(P)H-dependent yALRs, K^{RO}_m for D-glucose was greater than 1 M. Kinetic parameters could therefore not be determined separately with these enzymes; values for k_{cat}/K^{RO}_m only are given. Expressed in k_{cat}/K^{RO}_m terms, the derivatives (**4**)–(**8**) were reduced up to 3000-fold more efficiently than D-glucose, which corresponds to a free energy difference of up to 20 kJ/mol. The most significant portion of this apparent extra binding energy is derived from increasing (by chemical modification) the percentage of the free aldehyde in solution, which is 1.5% for the derivatives and 0.0026% for D-glucose [18] (Schemes 1b and 1c). Hence a 577-fold increase in k_{cat}/K^{RO}_m was predicted; this agreed in a satisfactory manner with the observations reported in Tables 3 and 4. Notably, the effects on k_{cat}/K^{RO}_m did not simply reflect tighter apparent binding of the glucose derivatives. The K^{RO}_m values of NADPH-dependent yALRs with compounds (**4**)–(**8**) were significantly larger than expected (compare Tables 3 and 4).

The active site of yALR seems to be completely permissive for accomodating and reducing D-glucose analogues in which the C-5 and C-6 hydroxy groups are replaced by hydrogen or fluorine. On permutation of these substituents at positions C-5 and C-6 to give compounds (**4**), (**6**), (**7**) and (**8**), the effects on the resulting kinetic parameters for aldehyde reduction by yALRs were small. In terms of differences in free energy, they were generally within a range of 1–2RT, reflecting a preference for the reduction of 5,6-dideoxy-D-glucose. However, the kinetic parameters for reduction of (**9**) were clear exceptions in the series of glucose derivatives. Introducing azide at C-6 of D-glucose led to a destabilization of the transition state for the yALR-catalysed reaction by approx. 7.3–11.7 kJ/mol, compared with for example the k_{cat}/K^{RO}_m values for (**8**) and (**9**) in Tables 3 and 4. Interestingly, fluorine introduced at C-5 could compensate almost completely for the destabilization of the transition state brought about by azide at C-6 [compound (**5**)]. Surprisingly, compounds (**4**) and (**6**), with fluorine at C-5 and fluorine or hydrogen at C-6 respectively, were not preferred to (**5**) by yALRs.

The results obtained with derivatives (**1**)–(**9**) suggest that the free aldehyde form of the aldose is the true substrate of yALR. In agreement with this notion, D-galactose (0.02% free aldehyde [18]) was a better substrate than D-glucose for aldehyde reduction catalysed by yALR (Tables 3 and 4). However, there is a weakly unfavourable contribution to catalytic efficiency of the C-4 (R) hydroxy group in D-aldoses [6], which certainly clouded the effect of the relative proportion of free aldehyde (Tables 3 and 4). Also of note is the 1.7–1.8-fold greater k_{cat} value with D-galactose compared with that with D-glucose.

NAD(P)H-dependent reduction of non-hydroxylated, cyclic and straight-chain aldehydes

We used CHCA (Scheme 1e) to determine whether the active site of yALR can interact productively with aldehydes of the form R-CHO, where R is a six-membered ring and structurally similar to the pyranose ring of sugars. The kinetic parameters for CHCA reduction are summarized in Tables 3 and 4 and compared with

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Table 3 Kinetic parameters for NADPH-dependent reduction of glucose derivatives and other aldehydes by yALR

The concentration of NADPH was 220 μM and saturating. Reaction conditions: 50 mM potassium phosphate buffer, pH 7.0, 25 °C. For K^{RO}_m and k_{cat}/K^{RO}_m the ratio is given (in parenthesis) of the observed value to the value expected on comparison of the percentage of free aldehyde form in aqueous solution of glucose (0.0026%), galactose (0.02%) or glucose derivatives (1.5%). $\Delta\Delta G$ was calculated from $\Delta\Delta G = -RT\ln[(k_{cat}/K^{RO}_m)_{deriv}/(k_{cat}/K^{RO}_m)_{xylose}]$; $\Delta\Delta G_{predicted} - \Delta\Delta G_{observed}$ is shown in parenthesis; $\Delta\Delta G_{predicted}$ was $-RT\ln[1.5/0.0026]$. Abbreviation: n.d., not determined.

	Cryptococcus flavus				*Candida intermedia* I			
Substrate	k_{cat} (s^{-1})	K^{RO}_m (mM)	k_{cat}/K^{RO}_m ($M^{-1}\cdot s^{-1}$)	$-\Delta\Delta G$ (kJ/mol)	k_{cat} (s^{-1})	K^{RO}_m (mM)	k_{cat}/K^{RO}_m ($M^{-1}\cdot s^{-1}$)	$-\Delta\Delta G$ (kJ/mol)
Glucose	3.4±0.1	100±3	34	–	5.1±0.2	264±20	19.3	–
5,6-Difluoro (**4**)	19±2	4.4±0.9 (25)	4318 (0.2)	12.0 (−3.8)	22±4	1.5±0.6 (3.3)	14667 (1.3)	16.4 (0.6)
6-Azido-5-fluoro (**5**)	14±1	0.6±0.2 (3.5)	23333 (1.2)	16.2 (0.4)	19±3	1.3±0.5 (2.8)	14615 (1.3)	16.4 (0.6)
6-Deoxy-5-fluoro (**6**)	14±1	0.4±0.1 (2.3)	35000 (1.8)	17.2 (1.4)	21±6	3.0±1.7 (6.6)	7000 (0.6)	14.6 (−1.2)
5-Deoxy-6-fluoro (**7**)	14±2	1.0±0.4 (5.8)	14000 (0.7)	14.9 (−0.9)	34±11	10.4±4.6 (22.7)	3269 (0.3)	12.7 (−3.1)
5,6-Dideoxy (**8**)	15±1	0.9±0.0 (5.2)	16667 (0.8)	15.4 (−0.4)	27±5	2.2±0.9 (4.8)	12273 (1.1)	16.0 (0.2)
6-Azido-5-deoxy (**9**)	n.d.	n.d.	900 (0.05)	8.1 (−7.7)	n.d.	n.d.	202 (0.02)	5.8 (−10.0)
Galactose	5.9±0.1	51±4 (3.9)	116 (0.4)	3.0 (−2.1)	9.4±0.6	303±22 (8.8)	31 (0.2)	1.2 (−3.9)
Oenanthaldehyde	9.2±0.6	0.6±0.1	15333	–	22±1	2.5±0.1	8800	–
CHCA	6.3±1.2	9.8±3.7	643	−7.9*	13.1±0.2	0.32±0.01	40938	3.8*

* Catalytic efficiency with oenanthaldehyde was used for the calculation.

Table 4 Kinetic parameters for NADH-dependent reduction of glucose derivatives and other aldehydes by yALR

The concentration of NADH was 220 μM and saturating. Reaction conditions: 50 mM potassium phosphate buffer, pH 7.0, 25 °C. For K^{RO}_m and k_{cat}/K^{RO}_m the ratio is given (in parenthesis) of the observed value to the value expected on comparison of the percentage of free aldehyde form in aqueous solution of glucose (0.0026%), galactose (0.02%) or glucose derivatives (1.5%). $\Delta\Delta G$ was calculated from $\Delta\Delta G = -RT\ln[(k_{cat}/K^{RO}_m)_{deriv}/(k_{cat}/K^{RO}_m)_{xylose}]$; $\Delta\Delta G_{predicted} - \Delta\Delta G_{observed}$ is shown in parenthesis; $\Delta\Delta G_{predicted}$ was $-RT\ln[1.5/0.0026]$. Abbreviation: n.d., not determined.

	Candida tenuis				*Candida intermedia* II			
Substrate	k_{cat} (s^{-1})	K^{RO}_m (mM)	k_{cat}/K^{RO}_m ($M^{-1}\cdot s^{-1}$)	$-\Delta\Delta G$ (kJ/mol)	k_{cat} (s^{-1})	K^{RO}_m (mM)	k_{cat}/K^{RO}_m ($M^{-1}\cdot s^{-1}$)	$-\Delta\Delta G$ (kJ/mol)
Glucose	n.d.	n.d	21	–	n.d.	n.d.	2.7	–
5,6-Difluoro (**4**)	18±1.9	1.4±0.6	12857 (1.1)	15.9 (0.1)	12±1	4.5±1.4	2667 (1.7)	17.1 (1.3)
6-Azido-5-fluoro (**5**)	16±0.3	0.7±0.1	22857 (1.9)	17.3 (1.5)	14±1	2.5±0.8	5600 (3.6)	18.9 (3.1)
6-Deoxy-5-fluoro (**6**)	18±0.5	1.2±0.2	15000 (1.2)	16.3 (0.5)	13±1	8.9±2.2	1461 (0.9)	15.6 (−0.2)
5-Deoxy-6-fluoro (**7**)	18±2.8	2.6±1.3	6923 (0.6)	14.4 (−1.4)	21±5	14.7±7.0	1428 (0.9)	15.5 (−0.2)
5,6-Dideoxy (**8**)	18±1.3	0.5±0.3	36000 (3.0)	18.5 (2.7)	14±1	1.7±0.5	8235 (5.3)	19.9 (4.1)
6-Azido-5-deoxy (**9**)	n.d.	n.d.	324 (0.03)	6.8 (−9.0)	n.d.	n.d.	148 (0.1)	9.9 (−5.9)
Galactose	12±1*	228±27	53 (0.3)	2.3 (−2.8)	5.9±0.1	74±4	80 (3.8)	8.4 (3.3)
Oenanthaldehyde	21±3	5.4±0.6	3888	–	4.6±0.2	4.7±1.1	979	–
CHCA	20±1	0.28±0.03	7143	1.5†	n.d.	n.d.	1081	~0†

* From [11].
† Catalytic efficiency with oenanthaldehyde was used for the calculation.

the kinetic parameters for oenanthaldehyde (Scheme 1d), which is the corresponding seven-carbon straight-chain aldehyde. CHCA was a substrate for all yALRs. However, the catalytic efficiencies for CHCA reduction varied within a 64-fold range and therefore revealed clear differences between individual yALRs. For NADPH-dependent ALR from *C. intermedia*, CHCA was reduced with a k_{cat}/K^{RO}_m that was significantly greater than k_{cat}/K^{RO}_m values for the reduction of the tightly binding D-glucose derivatives. Unlike the variation in kinetic parameters observed with CHCA, oenanthaldehyde was quite a good substrate for all yALRs, with k_{cat}/K^{RO}_m values in a 16-fold range. The k_{cat} values for the reductions of oenanthaldehyde and D-xylose were generally similar. The NAD(P)H-dependent enzyme from *C. intermedia* is an exception because k_{cat} values for oenanthaldehyde reduction, with either NADPH or NADH as coenzyme, were 1/2.3 times the corresponding turnover numbers with D-xylose.

DISCUSSION

Specificity of ALRs

The ALRs are known as broad-spectrum enzyme catalysts capable of reducing into the corresponding primary alcohols a wide variety of substrates with an aldehyde as the functional group [8]. However, the substrate-binding pockets of the ALRs so far characterized are clearly neither indiscriminate nor uniform in their ability to accommodate different aldehyde substrates. Furthermore, the active sites of ALRs are different in the extent to which they can use binding energy derived from non-covalent interactions between the enzyme and non-reacting portions of the aldehyde for transition state stabilization and rate enhancement. Therefore a detailed elucidaton of the relationships between substrate structure and reactivity towards reduction catalysed by different ALRs is expected to provide useful information about the evolution of enzyme function in the aldo/keto reductase

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superfamily. In addition, it will contribute to an understanding of the roles of individual enzymes *in vivo*, which for ALRs seem to range from detoxification metabolism [8] to mainstream carbohydrate catabolism [11]. The occurrence of multiple forms of aldose reductase in xylose-metabolizing yeasts, for example, is only partly understood in terms of its physiological importance. To address this question in some detail, we chose in a first approach to compare the substrate specificities of a representative selection of yALRs by using analogues of D-xylose and D-glucose as aldehyde probes.

Interpretation of apparent kinetic parameters

The ALRs so far studied operate by ordered kinetic mechanisms in which NAD(P)H binds before the aldehyde. For mALR, the slow forward isomerization of the enzyme–NADP complex before the NADP-dissociation step governs k_{cat} entirely and furthermore determines that k_{cat}/K^{RO}_{m} approximates the second-order rate constant for aldehyde binding to the enzyme [9,10]. Accordingly, K^{RO}_{m} reflects the ratio of the forward isomerization rate constant to the rate constant for aldehyde binding rather than being a true dissociation constant. For yALRs the full kinetic mechanism has not yet been determined; an unequivocal assignment of the kinetic parameters to microscopic rate constants is therefore not possible. However, comparison of typical k_{cat} values of yALR (10–20 s^{-1}) and mALR (0.1–1 s^{-1}) indicates that nucleotide exchange in yALR, independently of its contribution to rate limitation in the catalytic cycle of yALR, must occur at a rate approx. 10–200-fold faster than in mALR. A probable consequence of the faster nucleotide dissociation in yALR would be that the apparent binding constant, K^{RO}_{m}, of yALR can increase relative to the K^{RO}_{m} of mALR although no change in the true D-xylose binding constant must take place, of necessity. Differences in the apparent binding of D-xylose are indeed noted, with K^{RO}_{m} values of 1 and 30–80 mM for mALR and yALR respectively.

Binding of the acyclic aldehyde form

On the basis of kinetic arguments, Grimshaw [19] concluded that mALR acts in the NADPH-dependent reduction of D-glucose by trapping the small amount of free aldehyde that is present in aqueous solution of the hexose. The results obtained here provide compelling and direct evidence in support of this model of ALR action. A comparison of the catalytic efficiencies of yALRs for the NAD(P)H-dependent reduction (k_{cat}/K^{RO}_{m}) of a series of natural and chemically derivatized aldoses reflects almost quantitatively the relative proportions of the free aldehyde species in aqueous solution of these aldoses. For the aldoses used here, the percentage of free aldehyde spans a range of nearly 600-fold, from 0.0026% for D-glucose to approx. 1.5% for the aldose derivatives. The k_{cat} values of yALRs vary only slightly over a series of homologous aldehydes derived from D-xylose. Therefore changes in k_{cat}/K^{RO}_{m} express merely the effects on apparent binding (K^{RO}_{m}). Furthermore, the results imply that the thermodynamic stabilities of the cyclic and acyclic forms of the aldose make little, if any, contribution to the activation free energy of the rate-determining step in the catalytic cycle of yALRs, as expected of an enzyme that does not catalyse ring opening. The effects on k_{cat} that we see in the experiments are ascribed to non-productive binding of cyclic aldehydes; this is discussed below.

Role of hydrophobic bonding

The aldehyde-binding sites of yALR (from *C. tenuis*) and mALR are respectively approx. 1.4-fold and 2.4-fold more hydrophobic than n-octanol. Binding energy derived from hydrophobic interactions between enzyme–NAD(P)H and the aldehyde has been shown to contribute approx. 14 kJ/mol (yALR [11]) and 16 kJ/mol (mALR [20]) to stabilization of the transition state. A straight-chain aldehyde composed of seven carbon atoms is reduced by all yALRs with a catalytic efficiency significantly greater than the k_{cat}/K^{RO}_{m} for D-xylose, stressing the important role of substrate hydrophobicity in catalysis. An interesting difference between the ALRs from *C. intermedia* is brought out by comparing the kinetic parameters for the reaction with oenanthaldehyde. Although values of k_{cat}/K^{RO}_{m} for D-xylose are almost identical with those of both ALRs, reduction of the straight-chain aldehyde is accomplished by NADPH-dependent ALR with a catalytic efficiency approx. 10-fold that of the NAD(P)H-dependent enzyme. The NADPH-dependent ALR efficiently uses hydrophobic interactions with its substrate to decrease the activation free energy of the rate-limiting step, represented by the maximum value of k_{cat}. The NAD(P)H-dependent ALR needs additional probably hydrogen-bonding interactions with its substrate (see below) to achieve efficient turnover. In conclusion, the NAD(P)H-dependent ALR seems more specific than its strictly NADPH-dependent counterpart for reducing hydroxylated aldehyde substrates such as aldoses. The NADPH-dependent ALR, in contrast, seems to have acquired a greater catalytic flexibility than the NAD(P)H-dependent ALR and in this respect it resembles the ALRs from a mammalian source [8]. If *C. intermedia* ALRs do indeed have a physiological role in the reduction of aldehydes other than D-xylose, our analysis suggests that the NADPH-dependent yALR is adapted to accomplish such additional functions. Comparison of the kinetic parameters of the ALRs from *C. tenuis* and *Cr. flavus* provides further evidence in support of the notion that the NAD(P)H-dependent ALR has a greater dependence on specific interactions with the polyhydroxylated substrate to achieve catalytic efficiency.

Role of hydrogen-bonding

The results described here and previously [11] provide an estimate of the upper limit for the catalytic efficiency of yALR: it is approx. $(0.9–1.6)\times10^{6}\ M^{-1}\cdot s^{-1}$. By using the k_{cat}/K^{RO}_{m} values for the reaction with D-xylose (corrected for the free aldehyde form; Tables 1 and 2) and oenanthaldehyde (Tables 3 and 4), we can obtain an estimate of the total transition-state stabilization energy that is contributed by hydrogen-bonding interactions between the binary yALR–nucleotide complex and the natural substrate. It is 11.5 kJ/mol for the NADPH-dependent ALRs and approx. 14–17 kJ/mol for the NAD(P)H-dependent ALRs. Three or four hydrogen bonds between uncharged donor–acceptor pairs [21] could account for this amount of binding energy, which is therefore extremely small for carbohydrate-active enzymes. Note that previously an estimate of approx. 17 kJ/mol was made for the transition-state stabilization energy derived from the interaction of *C. tenuis* ALR with a single C-2 (*R*) hydroxy group of D-galactose [11].

It has been shown here, by using aldose derivatives in which the hydroxy groups at C-5 and C-6 had been replaced by hydrogen and fluorine, that yALR does not use hydrogen-bonding interactions with these hydroxy groups to bring about specificity. The conclusion rests on the widely accepted assumption [22] that the fluorine can accept but, of course, not

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donate a hydrogen bond, whereas hydrogen cannot participate in either interaction with the enzyme. Therefore, on basis of binding effects alone the deoxygenated compounds should generally be poorer substrates than the parent sugar. For the enzyme's functioning as the donor of a hydrogen bond to a particular sugar hydroxy group, the deoxy substrate should be worse than the deoxyfluoro one. Deoxy derivatives of xylose and glucose are on average equally good or even better substrates of yALR than the parent sugars. In NAD(P)H-dependent yALR, for example, the hydroxy group at C-6 of glucose makes a significant non-favourable contribution of 2.7–4.1 kJ/mol.

Recently, attempts have been made to identify important enzyme/aldehyde interactions in the active site of mALR (from bovine kidney) by using deoxy and deoxyfluoro derivatives of D-glucose [23]. An unexpected result of this study has been that 3-fluoro and 4-fluoro-D-glucose are, respectively, approx. 50-fold and 10-fold better substrates of mALR than is D-glucose. The observed effects are entirely due to an apparently better binding of the analogues. They cannot be explained in terms of hydrogen-bonding interactions of mALR with the substituents at C-3 and C-4. However, halomethyl groups are hydrophobic compared with the methyl and the hydroxymethyl group [24]. Therefore the results of Scott and Viola [23] could reflect the importance of hydrophobic bonds between the substrate and mALR at the particular carbon atoms. Assuming that fluoro sugars are more hydrophobic than the corresponding deoxy sugars, the preference of yALRs for compounds (**2**) relative to (**3**), and (**8**) relative to (**4**), seems surprising. However, if the fluoro-substituted sugar hydrogen bonds as acceptor with water, there will be a net loss of hydrogen bonds on transfer of the sugar to the active sites of yALRs. In such a situation the deoxy sugar will have a greater affinity than the fluoro sugar, accounting reasonably for the experimental observations with yALRs.

The effect of azide introduced at terminal carbon positions of the substrate is a loss of binding energy of 6–10 kJ/mol. Azide is a dipolar molecule and a good nucleophile. Therefore its transfer from water to a non-polar environment such as the active site of ALR is likely to be unfavourable. Its negative contribution to transition-state binding by yALRs matches the unfavourable incremental Gibbs free energy expected for the transfer of a hydroxy group (attached to aliphatic compounds) from water to n-octanol.

Non-productive binding

This is known to be used by enzymes as a mechanism to prevent the rapid reaction of poor substrates to products [25]. Non-productive binding brings about specificity with only little expediture of binding energy of good and specific substrates, although it does not help catalysis as such. When the non-productive enzyme–substrate complex is completely inactive, the observed effective turnover number, k_{cat} (eff), will be decreased by the fraction of productively bound substrate, F, i.e. k_{cat} (eff) $= k_{cat}F$. In contrast, the second-order rate constant will not be affected by non-productive binding. Specificity achieved via non-productive binding can be described as a free energy, $\Delta G = -RT\ln F$.

All yALRs are active with CHCA, an aldehyde in which the non-reacting part is a cyclohexane ring in the chair conformation. Therefore the binding of aldopyranoses to the active sites of yALRs will not be excluded simply because of steric hindrance. However, if it occurred it would certainly be non-productive. With NADPH-dependent yALRs, the comparison of the kinetic parameters observed with D-glucose and D-glucose analogues (**4**)–(**8**) strongly suggests non-productive binding of D-glucose as a pyranose ring. The k_{cat} values with (**4**)–(**8**) are up to 5.5-fold the corresponding catalytic-centre activity with D-glucose, that is $F \approx 0.18$ (1/5.5). Judging from the differences in the proportion of free aldehyde in aqueous solution, the $K^{RO}{}_{m}$ for D-glucose is decreased relative to the corresponding $K^{RO}{}_{m}$ values for glucose analogues by about the same factor. The free energy that discriminates against glucose in these enzymes is approx. 4.2 kJ/mol, that is $RT\ln(1/5.5)$, and 5 kJ/mol, that is $RT\ln(0.0026/0.02)$, accounting for the different percentage of free aldehyde present in solutions of D-glucose and D-xylose. Although this energy is indeed quite small, it is enough to prevent the yALR-catalysed reduction of glucose almost completely in the presence of equimolar D-xylose (0.1–0.3 M) when internal recycling of NADPH or NADH (0.5 mM) is performed with the formate dehydrogenase-catalysed oxidation of formate anion (B. Nidetzky and P. Mayr, unpublished work). In conclusion, extra specificity to discriminate against D-glucose is achieved by yALR via non-productive binding, decreasing k_{cat} (and $K^{RO}{}_{m}$) of NADPH-dependent ALRs, and unfavourable interactions with the C-6 hydroxy group, increasing $K^{RO}{}_{m}$ of the NAD(P)H-dependent ALRs.

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