Noncovalent Enzyme−Substrate Interactions in the Catalytic Mechanism of Yeast Aldose Reductase[†]

Wilfried Neuhauser, Dietmar Haltrich, Klaus D. Kulbe, and Bernd Nidetzky*

Division of Biochemical Engineering, Institute of Food Technology, Universität für Bodenkultur (BOKU), Muthgasse 18, A-1190 Wien, Austria

Received July 22, 1997; Revised Manuscript Received October 31, 1997

ABSTRACT: The role of noncovalent interactions in the catalytic mechanism of aldose reductase from the yeast *Candida tenuis* was determined by steady-state kinetic analysis of the NADH-dependent reduction of various aldehydes, differing in hydrophobicity and the hydrogen bonding capability with the binary enzyme·NADH complex. In a series of aliphatic aldehydes, substrate hydrophobicity contributes up to 13.7 kJ/mol of binding energy. The aldehyde binding site of aldose reductase appears to be 1.4 times more hydrophobic than *n*-octanol and can accommodate a linear alkyl chain with at least seven methylene groups (≈14 Å in length). Binding energy resulting from interactions at positions 3−6 of the aldehyde is distributed between increasing the catalytic constant 2.6-fold and decreasing the apparent dissociation constant 59-fold. Hydrogen bonding interactions of the enzyme·nucleotide complex with the C-2(*R*) hydroxyl group of the aldehyde are crucial to transition state binding and contribute up to 17 kJ/mol of binding energy. A comparison of the kinetic data of yeast aldose reductase, a key enzyme in the metabolism of D-xylose, and human aldose reductase, a presumably perfect detoxification catalyst [Grimshaw, C. E. (1992) *Biochemistry 31,* 10139], clearly reflects these differences in physiological function.

Aldose reductase [alditol:NAD(P) oxidoreductase, EC 1.1.1.21] (ALR),[1] a member of the aldo/keto reductase superfamily, catalyzes the NAD(P)H-dependent reduction of aldehydes to their corresponding alcohols with very broad specificity. The enzyme is widely distributed in nature and occurs as a predominantly monomeric, approximately 35-kDa protein in mammalian tissues, plants, and microorganisms such as fungi and yeasts. The current understanding of the catalytic mechanism of ALR rests on the biochemical and structural studies on mammalian ALRs (*1*−*3*), and it is clear that there is an overall structural relationship between ALRs from mammals and microbes (*4*−*6*). Mammalian ALR contains an α_8/β_8-barrel structural motif, to which NADPH binds in an extended conformation (*7*), and has a deep, very hydrophobic substrate binding pocket at the COOH-terminal end of the barrel (*7*−*9*). The hALR, like the related aldehyde reductase (*10*), stereospecifically transfers the 4-*pro-R* hydrogen, in the form of the hydride ion, from the C4 of the nicotinamide ring to the *re* face of the carbonyl carbon of the substrate (*1*). The active site contains Tyr48 as the proton donor during aldehyde reduction whereas His110 is important for the orientation of the substrate in the substrate binding pocket (*2, 11*). The pK_a of Tyr48 is effectively perturbed by the adjoining Asp43/Lys77 residues, resulting in an apparent shift of 2 pK units for this tyrosine from 10.5 to 8.4 (*12*). The catalytic residues are all completely conserved in yeast ALRs (*5, 6, 13*), so that it is safe to assume that yeast ALR will follow the same catalytic mechanism as hALR. Modification of one cysteine residue (Cys298) in hALR results in enzyme forms that display altered kinetic properties (*14*−*17*). Interestingly, this cysteine is not conserved in the yeast enzymes (*5, 6, 13*), and, probably, the ALR from *Candida tenuis* is therefore not sensitive to oxidative modification (*18*). The kinetic mechanism of human, porcine, and yeast ALR was shown to be compulsory ordered with coenzyme binding first and leaving last (*15, 18*−*21*). The turnover number in aldehyde reduction by hALR is limited by an enzyme·NADP isomerization step that precedes the release of nucleotide (*3*). From crystallographic analyses of the ALR apoenzyme (*9*) and holoenzyme (*7, 8*), it is clear that the movement of a nucleotide binding loop is required for coenzyme binding and release. Unfortunately, binding of the aldehyde substrate in the active site of the ALR crystal has not been observed yet, but has been studied by molecular modeling (*11, 22*).

The physiological functions of hALR are thought to include the detoxification of a wide range of reactive carbonyl-containing metabolites (*23, 24*) and the formation of intracellular polyol as a mechanism to counteract negative osmotic effects (*25*). Under normal physiological conditions, the hALR-mediated aldehyde reduction does not represent a 'high-flux' metabolic route, and the kinetic properties of hALR seem to be in contrast to wideheld tenets of enzymic perfection (*26*−*28*) and use of binding energy for efficient catalysis (*29*). Grimshaw (*24*) outlined elegantly that ALR has probably evolved to enzymic perfection as a detoxifi-

[†] Supported by the Austrian Bundesministerium für Land- und Forstwirtschaft (Grant 844/94) and the Austrian Science Foundation (Grant P-12569).

* Correspondence should be addressed to this author at the Institute of Food Technology, Muthgasse 18, A-1190 Wien, Austria. Telephone: (+43)-1-36006-6274. Fax: (+43)-1-36006-6251. E-mail: nide@mail.boku.ac.at.

[1] Abbreviations: hALR, human aldose reductase; yALR, yeast aldose reductase (from *Candida tenuis*).

S0006-2960(97)01780-7 CCC: $15.00
Published on Web 01/06/1998

cation catalyst by acting with low turnover numbers, but comparable and high catalytic efficiencies, with a wide variety of substrates containing an aldehyde as functional group. Transition-state stabilization by noncovalent interactions of the enzyme with nonreacting portions of the aldehyde substrate is thus very small. The hALR binds the coenzyme, NADPH, extremely tightly, with a dissociation constant $\leq 0.05\ \mu$M, such that ALR is saturated with nucleotide at all times to generate the reactive binary enzyme·NADPH complex (*24*). The enzyme apparently derives most of the energy required for transition state stabilization from the tight interaction with the coenzyme.

In contrast to hALR, the ALR from yeast is a key metabolic enzyme that catalyzes the first step in the pathway of D-xylose utilization, thus reducing D-xylose to xylitol in a NADPH- or NADH-dependent manner (*30*−*32*). Hence, this ALR is required for growth and energy, reflected, e.g., by the fact that the enzyme in yeast is inducible by D-xylose (*33*). It therefore seems interesting to pinpoint differences in the kinetic properties of mammalian and yeast ALR that are expected to reflect the differences in physiological function, at least to some extent. Compared to hALR, noncovalent interactions between the binary enzyme·nucleotide complex and the substrate aldehyde could be more important with yALR, and this aspect of the catalytic mechanism of yALR has not been investigated. Noncovalent interactions are thought to be crucial to enzyme catalysis (*29*), and considering the spectrum of aldehydes reduced by ALR (e.g., *15*, *18*, *34*), it seems probable that enzyme−substrate interactions for ALR would involve hydrogen bonds as well hydrophobic bonds.

In this work, we show that the ALR from the yeast *Candida tenuis* can use binding energy derived from binding of the aldehyde substrate for a significant transition state stabilization. The binding energy results predominantly from (i) hydrogen bonding interactions of ALR with the hydroxyl group at the 2 position of the aldehyde ($\approx$17 kJ/mol) and (ii) hydrophobic bonding interactions at the ALR substrate binding pocket ($\approx$14 kJ/mol). The C-2 hydroxyl group functions as acceptor and probably as donor of hydrogen bonds. The results are rationalized in terms of the metabolic function of yeast ALR in D-xylose utilization as opposed to the role of mammalian ALR as an aldehyde-removing catalyst in cell detoxification.

MATERIALS AND METHODS

Materials. If not mentioned otherwise, all chemicals were of the highest purity available and obtained from Sigma and Fluka. Aliphatic aldehydes of the form R-CHO, where R is the alkyl chain with 3−8 carbon atoms (purity $\geq$98%; content of R-COOH $\leq$2%), were stored in sealed bottles, and aqueous solutions of these aldehydes were made immediately prior to use. Derivatives of D-galactose (2-deoxy, 2-fluorodeoxy, 3-deoxy, 6-deoxy) were from Chemprosa (Graz, Austria). The 3,6-anhydro-D-galactose was from Dextra Laboratories (U.K.). D-*Arabino*-hexosulose (D-glucosone), D-*ribo*-hexosulose (D-allosone), D-*lyxo*-hexosulose (D-galactosone), and D-*threo*-pentosulose (D-xylosone) were prepared by the complete conversion of the corresponding D-aldoses (300 mM; 30 °C) using pyranose oxidase as described (*35*). The resulting 2-keto-aldoses were employed for kinetic studies with yALR following purification by anion exchange chromatography on DEAE-Sepharose, gel filtration on Sephadex G25 c, and concentration by freeze-drying. Judged from analysis by TLC and HPLC, the products were $\geq$99% pure. The 5-deoxy-D-xylose, synthesized by reported methods (*36*), was a kind gift of Dr. A. Stütz (Technical University Graz, Austria).

Enzymes. The ALR from the yeast *Candida tenuis* CBS 4435 was produced and purified as described previously (*18*).

Initial Velocity Studies and Kinetic Analyses. The ALR from *C. tenuis* is able to use with comparable specificity NADPH and NADH for aldehyde reduction (*18*). Hence, NADH was used as coenzyme throughout this study. All measurements were performed with a Beckman DU-65 spectrophotometer. ALR activity was assayed at 25 °C monitoring the oxidation of NADH at 340 nm (1−5 min, rate of 0.05−0.1 $\Delta A \cdot \text{min}^{-1}$) in the direction of aldehyde reduction. All rates were corrected for the appropriate blank readings, lacking either the substrate or the enzyme. The standard reaction mixture (1-mL volume) contained 15−150 nM yALR and a constant, saturating concentration of 300 μM NADH (apparent K_m of 15 μM; *18*) in 50 mM potassium phosphate, pH 7.0. The aldehyde substrate was varied, typically in 7−10 different concentration points, over a concentration range covering approximately from 0.1 to 5−10 times the K_m for each aldehyde, and each point (initial velocity) was determined in triplicate. If not mentioned otherwise, apparent saturation was achieved with all substrates, and at least 3 concentration points in the region of the K_m were measured. All kinetic parameters were calculated by fitting the Michaelis−Menten function (eq 1) directly to the data (SigmaPlot, version 5) using an unweighted nonlinear least-squares analysis:

$$V(\text{RO}) = k_{cat}[\text{E}][\text{RO}]/(K_{m\ \text{RO}} + [\text{RO}]) \quad (1)$$

In cases where substrate inhibition occurred, eq 1 was modified to eq 2:

$$V(\text{RO}) = k_{cat}[\text{E}][\text{RO}]/(K_{m\ \text{RO}} + [\text{RO}] + [\text{RO}]^2/K_{is}) \quad (2)$$

where V(RO) is the initial velocity, k_{cat} is the catalytic constant, [E] is the total concentration of yALR, [RO] is the varied substrate concentration, $K_{m\ \text{RO}}$ is the apparent Michaelis constant for RO, and K_{iS} is the substrate inhibition constant for RO. In the case of some aromatic aldehydes that are poorly soluble in water (e.g., chlorobenzaldehyde), the catalytic efficiency for aldehyde reduction by yALR, $k_{cat}/K_{m\ \text{RO}}$, was derived from the linear part of the Michaelis−Menten curve, when at nonsaturating concentrations of aldehyde the reaction rate is linearly dependent on the substrate concentration. It was also proven that concentrations of free aldehyde greater than 0.5 M did not inactivate yALR, for example, by modification of lysine residues, during a 1-h incubation at 30 °C.

RESULTS

Aliphatic Aldehydes as Substrates of Yeast Aldose Reductase. Aldehydes of the form R-CHO, where R is an unbranched alkyl chain, were used as substrates for the NADH-dependent reduction by ALR. The kinetic parameters change with increasing hydrophobicity of R (Table 1),

Table 1: Apparent Kinetic Parameters of Yeast ALR with Aliphatic Aldehydes of the Form R-CHO, Where R Is the Alkyl Chain[a]

alkyl chain of R	π[b]	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	$-\Delta\Delta G$ of k_{cat}/K_m (kJ/mol)
$-CH_2-CH_3$	1.0	365.0	13.0	36	–
$-(CH_2)_2-CH_3$	1.5	63.6	28.3	445	6.2
$-(CH_2)_3-CH_3$	2.0	11.6	32.6	2100	10.1
$-(CH_2)_4-CH_3$	2.5	6.2	34.0	4838	12.1
$-(CH_2)_5-CH_3$	3.0	5.4	33.3	7500	13.2
$-(CH_2)_6-CH_3$	3.5	2.4	21.6	9136	13.7
$-(CH_2)_7-CH_3$	4.0	1.5	11.0	7189	13.1

[a] Reactions were carried out at 25 °C, pH 7.0, using a saturating concentration of 300 μM NADH. The enzyme concentration was 15 nM. Standard deviations <15%. [b] Hydrophobicity coefficient of R, relative to the hydrogen atom (*29*).

but there is a differential effect on the catalytic constant, k_{cat}, the apparent binding constant, K_m, and the specificity constant, k_{cat}/K_m. The values of K_m decrease 243-fold as R changes from $-CH_2CH_3$ to $-(CH_2)_7CH_3$ (Table 1). The substrate binding pocket of ALR can therefore accommodate the aldehyde group plus at least seven additional methylene groups, and, assuming a carbon−carbon distance of 1.55 Å, the estimated length of this binding pocket is ≥14 Å and hence well-comparable in size to the aldehyde binding pocket of the hALR (*7*; ≈ 12 Å). The binding energy that is derived from hydrophobic interactions of the ALR·nucleotide complex with the aldehyde at positions 3−6 is distributed between a 2.6-fold increase in k_{cat} and a 59-fold decrease in K_m. In contrast, favorable interactions at positions 7−9 of the substrate seem to be expended exclusively to lower the apparent K_m rather than the activation energy of reaction (Table 1). As evident from the variation of the catalytic constant with changes of R (Table 1), the occupancy of the substrate binding pocket by the aldehyde plus an alkyl rest of at least $-(CH_2)_2CH_3$ is required for efficient turnover. A further increase of the hydrophobicity of R has little effect on k_{cat}. For R ≥ $-(CH_2)_5CH_3$, the k_{cat} values decrease. The incremental Gibbs free energies of transfer of R from the enzyme to water, $-\Delta\Delta G_b$, were calculated relative to $-CH_2CH_3$, according to

$$\Delta\Delta G_b = -RT \ln [(k_{cat}/K_m)_{(CH_3-(CH_2)_x-CHO)}/(k_{cat}/K_m)_{(CH_3-CH_2-CHO)}]$$

where x is the number of methylene groups of R. The total binding energy contributed by the hydrophobic interaction with an aliphatic aldehyde is estimated to be approximately 14 kJ/mol (Table 1). When the values of $-\Delta\Delta G_b$ are plotted against the incremental Gibbs free energy of transfer of the group R from *n*-octanol to water, according to $2.303RT\pi$ (*29*), a linear relation is observed for the range of R from 2 to 5 carbons (Figure 1). The slope of the solid line in Figure 1 is 1.41, and thus the substrate binding pocket of ALR appears to be approximately 1.4 times more hydrophobic than *n*-octanol.

The reduction of K_m by a factor of 2.4 log units with increasing hydrophobicity of the aldehyde (Table 1) could point out the contribution of hydrophobic interactions to ground state binding of the aldehyde substrate. The pattern of inhibition of the ALR-catalyzed reduction of D-xylose by a series of carboxylic acids, R-COOH, where R is again an unbranched alkyl chain, seems to corroborate this notion. Carboxylic acids act as noncompetitive, rather than competitive inhibitors with respect to the aldehyde substrate, D-xylose, whereas in alcohol oxidation they act as competitive inhibitors with respect to xylitol (not shown). However, it is obvious from Figure 2 that the degree of inhibition (with respect to D-xylose) is a function of the hydrophobicity of the carboxylic acid. The inhibition in response to the hydrophobic character of R is biphasic (Figure 2). For R ≤ $-(CH_2)_3CH_3$ and for R ≥ $-(CH_2)_3CH_3$, the inhibitory competence is a linear function of the hydrophobicity of R. Interestingly, the inhibition by carboxylic acids composed of 4 or more carbons is significantly stronger than by those with a shorter alkyl chain. Hydrophobic bonding interactions at a position ≥C-4 thus seem to contribute especially to the ground state binding, rather than the transition state binding, of an aliphatic aldehyde to the ALR·nucleotide complex.

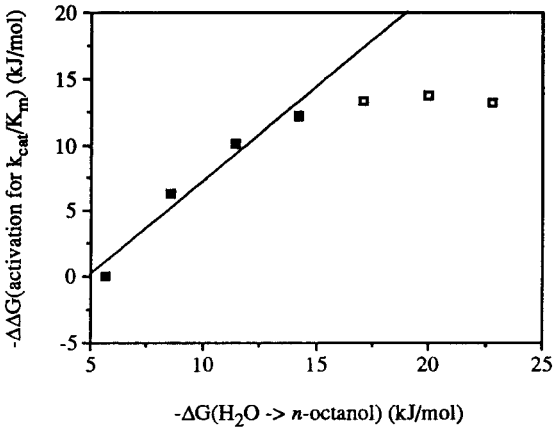

FIGURE 1: Relationship between the hydrophobicity of the aldehyde side chain, R, and the catalytic efficiency for the NADH-dependent reduction of the aldehyde by yALR. Data with full symbols were used for linear regression analysis.

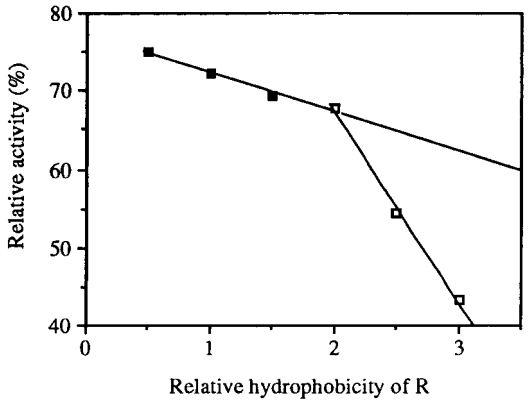

FIGURE 2: Relationship between the hydrophobicity of the side chain, R, of a carboxylic acid, R-COOH, and the inhibition of the yALR-catalyzed reduction of D-xylose. The concentrations of R-COOH, D-xylose, and NADH were 300 mM, 700 mM, and 300 μM, respectively. Reactions were carried out at 25 °C in 50 mM potassium phosphate buffer, pH 7.0.

Aromatic Aldehydes as Substrates of Yeast Aldose Reductase. Aldehydes of the form R-CHO, where R is a nonsubstituted or substituted aromatic ring, are known to comprise several excellent substrates for the ALRs from a mammalian source (*15*, *34*). The catalytic efficiencies for the NADH-dependent, ALR-catalyzed reduction of aromatic aldehydes, employed in nonsaturating concentrations of 0.1−

Table 2: Apparent Kinetic Parameters of Yeast ALR with Polyhydroxylated Aldehydes[a]

substrate	K_m (mM)	k_{cat} (s^{-1})	$[k_{cat}/K_m]$ (M^{-1} s^{-1})	$(k_{cat}/K_m)' \times 10^{-3}$ (M^{-1} s^{-1})
DL-glyceraldehyde	2.0	15.5	7760	32
D-erythrose	1.6	36.4	22189	554
D-xylose	87.0	18.2	211	1055
L-arabinose	27.0	13.0	480	1600
D-ribose	217	8.2	39	78
L-lyxose	681	10.9	16	53
D-glucose[c]	≈9000	26.0	4	20
D-galactose	228	14.7	66	330

[a] Reactions were carried out at 25 °C, pH 7.0, using a saturating concentration of 300 μM NADH. The enzyme concentration was 15 nM. Standard deviations <15%. [b] $[k_{cat}/K_m]' = f[k_{cat}/K_m]$; *f*, correction for the free aldehyde in solution (*24*, *38*). [c] Determination of kinetic parameters hampered because of strong uncompetitive substrate inhibition (inhibition constant $\ll K_m$).

10 mM, were measured and are expressed relative to the physiological substrate, D-xylose. All catalytic efficiencies with aromatic aldehydes were significantly higher than that observed with D-xylose: pyridine-2-carbaldehyde (6.6-fold), pyridine-3-carbaldehyde (3.3-fold), pyridine-4-carbaldehyde (6.7-fold), 3-nitrobenzaldehyde (5.8-fold), and 3-chlorobenzaldehyde (4.5-fold). However, when correction is made for the free aldehyde species of D-xylose in solution (see below and Table 2), the catalytic efficiencies are 700−1500-fold higher for the aldopentose than for the aromatic aldehydes. It is noteworthy that enzyme activity was observed only when R was a six-membered ring. In contrast, when R was a five-membered ring, like in pyrrole-2-carbaldehyde, furane-2-carbaldehyde, and thiophene-2-carbaldehyde, no reaction was observed. The ALR-catalyzed reduction of D-xylose was not inhibited by one of the aromatic five-ring aldehydes, indicating that these components do not even bind to the binary ALR·nucleotide complex. In contrast, 1-formylpiperidine (*12*), a six-ring aldehyde, was a competitive inhibitor with respect to D-xylose, with an inhibition constant of 150 mM.

Polyhydroxylated Aldehydes as Substrates of Yeast Aldose Reductase. The apparent kinetic parameters for aldehyde reduction were determined with a series of polyhydroxylated aldehydes (aldoses), composed of 3−6 carbon atoms (Table 2). The variation of the catalytic efficiency spans almost 4 log units, reflecting a 4.4-fold variation in k_{cat} and a 425-fold variation in the Michaelis constant for the aldehyde (the K_m for D-glucose of 9 M was not considered). However, there is good evidence that the free aldehyde species is the actual substrate of ALR (*37*; this work), and so a correction for the amount of free aldehyde in solution is necessary for a direct comparison of the kinetic parameters, shown as $(k_{cat}/K_m)'$ values in Table 2. The 17.3-fold increase in catalytic efficiency for D-erythrose, relative to DL-glyceraldehyde, corrborates the results obtained with aliphatic aldehydes (cf. Table 1) indicating that four 'subsites' in the ALR substrate binding pocket must be occupied to achieve fast turnover. The specificity constant, $(k_{cat}/K_m)'$, is highest with L-arabinose and the physiological substrate, D-xylose. As with human hALR but to a much higher extent, specific steric requirements for the hydroxyl group at the 2 position of the substrate were observed with the yeast enzyme. The C-2(*R*) configuration is of crucial importance to transition state binding so that substrates with the inverted, C-2(*S*), configuration such as L-xylose, D-arabinose, D-lyxose, D-mannose, L-glucose, and L-rhamnose were reduced with a more than 1000-fold reduced catalytic efficiency, relative to D-xylose. This corresponds to a loss of at least 17 kJ/mol of binding energy. A similar estimate of the contribution of the C-2 hydroxyl group was made from the specificity constants of DL-glyceraldehyde, based on free aldehyde species (Table 2), and propionaldehyde (Table 1), when it is assumed that the hydroxyl group of the primary alcohol at C-3 of DL-glyceraldehyde will not show notable interactions. Accordingly, hydrogen bondings at C-2 contribute approximately 16.8 kJ/mol of binding energy.

A weak, but significant preference of yeast ALR for the configuration C-3 (*S*), in D-xylose compared to D-ribose, and C-4(*S*), in L-arabinose compared to D-xylose or in D-ribose compared to L-lyxose, was observed, with an apparent contribution of 6.4 and 1.0 kJ/mol, respectively (Table 2). A drastic decrease in catalytic efficiency for the reduction of D-galactose is noted relative to the corresponding aldopentose, L-arabinose. The loss of binding energy of 3.9 kJ/mol for D-galactose suggests nonfavorable interactions of the enzyme·nucleotide complex with the C-5(*R*) hydroxyl group of the aldehyde.

Many interactions of ALR with polyhydroxylated substrates are likely to involve hydrogen bonds. Estimates of the contribution of hydrogen-bonding interactions at the transition state for aldehyde reduction have been obtained by kinetic studies using a series of derivatives of D-galactose in which the hydroxyl groups were substituted by hydrogen and, in the case of the 2 position, by fluorine (Table 3). The decrease in catalytic efficiency for the reduction of 2-deoxy-D-galactose was more than 1000-fold compared to D-galactose, thus again highlighting the important role of hydrogen-bonding interactions at C-2 of the substrate (≤17 kJ/mol). In contrast, the catalytic efficiency for the reduction of the 2-deoxy-2-fluoro derivative was only 6-fold lower than that with the parent sugar, D-galactose. This reduction corresponds to an overall loss of 4.4 kJ/mol of binding energy. Interestingly, the effect is due to a large, 15-fold reduction in k_{cat}, whereas aldehyde binding seems even tighter with the substituted derivative. The substitution of the hydroxyls with hydrogen at C-3 and C-6 of D-galactose had little effect on the corresponding catalytic efficiencies (Table 3). Hence, the C-3(*S*) hydroxyl group, e.g., in D-galactose, L-arabinose, and D-xylose, does by itself not contribute to binding energy via hydrogen-bonding interactions with ALR. However, nonfavorable interactions with the C-3(*R*) hydroxyl, e.g., in D-ribose and L-lyxose, are avoided in these substrates. The primary alcohol at C-6, in turn, seems not to be involved in any enzyme/substrate interaction involving hydrogen bonds, and, as shown for aliphatic aldehydes (Table 1), the contribution to binding energy of hydrophobic interactions at this position is very small, too.

Interaction of Aldose Reductase with the Open-Chain Aldehyde Form of Aldoses. Grimshaw (*37*) used bovine kidney ALR to measure the rate constants of ring opening of D-glucose and concluded that the enzyme does not by itself catalyze the ring-opening reaction and acts as reagent trapping the free carbonyl form of the sugar. However, kinetic studies with analogs of sugars that, relative to the parent compounds, contain a significantly increased fraction of open-chain aldehyde species in aqueous solution have to

Table 3: Apparent Kinetic Parameters of Yeast ALR with Analogs of D-Galactose and D-Xylose[a]

substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	$-\Delta\Delta G$ (kJ/mol)
D-galactose	228	14.7	66	–
2-deoxy-D-galactose			<0.06	>−17
2-deoxy-2-fluoro-D-galactose	92	1.0	11	−4.41
3-deoxy-D-galactose	127	9.7	76	0.35
6-deoxy-D-galactose	198	15.1	77	0.38
3,6-anhydro-D-galactose	0.6	8.3	13833	13.25
(acyclic aldehyde form)	(0.14)[b]		(57043)	(16.76)
D-xylose	87	18.2	211	–
5-deoxy-D-xylose (no pyranose form, ≈4% free aldehyde)	1.1	26.9	24455	11.8

[a] Reactions were carried out at 25 °C, pH 7.0, using a saturating concentration of 300 μM NADH. The enzyme concentration was 15 nM, or 150 nM with poor substrates (k_{cat} < 10 s^{-1}). Standard deviations are <15%. [b] Correction for α-hydroxyaldehyde hydration (*24*).

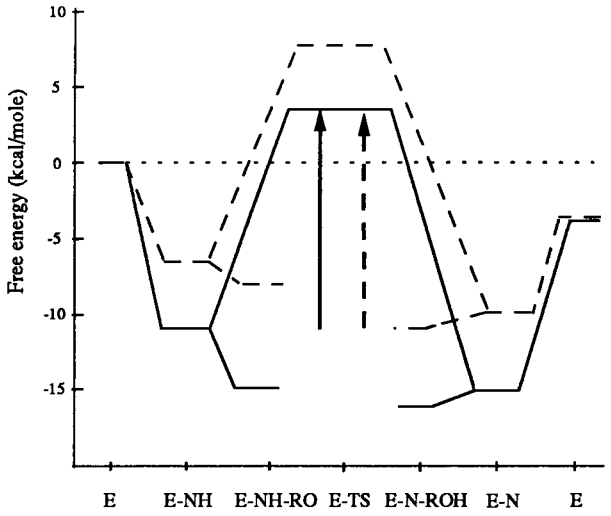

FIGURE 3: Reaction coordinate diagram comparing the NAD(P)H-dependent reduction of D-xylose by human (solid line) and yeast (dashed line) aldose reductase, where E is enzyme, NH and N are NAD(P)H and $NAD(P)^+$, RO and ROH are D-xylose and xylitol, and TS is the transition state, respectively. The arrows show $\Delta G^{\ddagger}$ values for the action of the human (solid) and yeast (dashed) enzyme. See Table 5 for the individual rate and equilibrium constants.

the best of our knowledge not been carried out. The kinetic parameters for reduction by yALR of intrinsically 'acyclic aldehyde' analogs of D-galactose, 3,6-anhydro-D-galactose, and 5-deoxy-D-xylose were determined and used for a comparison with those for reduction of D-galactose and D-xylose, respectively. The equilibrium composition of 5-deoxy-D-xylose in solution lacks the pyranose species, and, analogous to D-erythrose, it will probably contain a 100−200-fold greater fraction of the free aldehyde species than that of D-xylose, existing predominantly (>99%) in pyranose form. Compared to D-xylose, the catalytic efficiency for the analog is 115-fold higher (Table 3), thus accounting for an extra binding energy of 11.8 or 19.7 kJ/mol when corrected for the free aldehyde species. The latter value is already close to a value of 21 kJ/mol, predicted theoretically from the 0.02% fraction of open-chain aldehyde in an aqueous solution of D-xylose or D-galactose. The kinetic data for the open-chain derivative of D-galactose indicate a 200-fold increase of catalytic efficiency, relative to the corresponding parent sugar. Probably because of adverse effects by modification of D-galactose, the gain in binding energy for the analog is only 13 or 16.8 kJ/mol with correction for hydration. Interestingly, substrate inhibition was strong with 3,6-anhydro-D-galactose (K_{iS} 15 mM), but absent with D-galactose.

Table 4: Apparent Kinetic Parameters of Yeast ALR with Various 2-Oxoaldehydes[a]

substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	$-\Delta\Delta G$ (kJ/mol)
D-xylosone	4.6	19.1	4154	7.4
D-glucosone	5.1	23.9	4684	(17.5)
D-galactosone	5.3	21.2	4004	10.2
D-allosone	6.4	22.5	3520	nd[b]
phenylglyoxal	4.2	14.9	3540	–
methylglyoxal	6.3	26.3	4176	−5.0[c]
				11.8[d]

[a] Reactions were carried out at 25 °C, pH 7.0, using a saturating concentration of 300 μM NADH. The enzyme concentration was 15 nM. Standard deviations are <15%. [b] nd, not calculated because catalytic efficiency with D-allose was <0.1% of that of D-xylose. [c] Relative to DL-glyceraldehyde (Table 2). [d] Relative to propionaldehyde (Table 1).

Interaction of Aldose Reductase with 2-Oxoaldehydes. The substitution of the hydroxyl group by a carbonyl group at the 2 position of an aldose yields aldos-2-uloses, so-called 'osones'. Aldos-2-uloses have been implicated in the sugar-induced cross-linking of proteins, thus causing tissue damage, and are thought to be physiological targets of the action of mammalian ALRs (*39, 40*). When used as substrates of yeast ALR, a drastic increase in the catalytic efficiencies, relative to the corresponding aldose sugars without correction for free aldehyde, was observed. The apparent decrease in the activation energy for k_{cat}/K_m is in the range of 7−10 kJ/mol (Table 4). According to Vuorinen and Serriani (*41*), the free aldehyde species of xylosone in solution will be very low (<1%). However, because a proportion at equilibrium of 0.5% free aldehyde for D-xylosone would still be 25 times higher than that for D-xylose, the kinetic parameters of the aldosones and the parent aldoses have to be compared with caution. Substrate specificity, determined from a comparison of catalytic efficiencies in Table 4, reveals a different picture than that obtained from the kinetic data for the aldoses in Table 2. It appears that enzyme−substrate interactions at positions other than C-2 are not significantly contributing to binding energy for the series of osone substrates in Table 4. Other dicarbonyl compounds, methylglyoxal and phenylglyoxal, are reduced with similar catalytic efficiency as the aldos-2-uloses.

Comparison of the Kinetic Properties of Yeast and Human ALR. A comparison of the kinetic parameters of yeast and human ALR for the NADH- and NADPH-dependent reduction of D-xylose, respectively, is shown in Table 5 and as a free energy diagram in Figure 3, where a standard free energy of ALR, nucleotide, and D-xylose equal to zero and a 1.0 M standard state for substrates and products were assumed. In Figure 3, E is the enzyme, NH and N are NAD(P)H and $NAD(P)^+$, respectively, and RO and ROH are D-xylose and xylitol, respectively. Marked differences between the two enzymes are found at the free energies of the binary enzyme·nucleotide complexes and the ternary enzyme·nucleotide·aldehyde complexes. The binary E−NH and E−N complexes of yALR are destabilized by 4.4 and 6.1 kcal/mole

Table 5: Comparison of the Kinetic Parameters of Aldose Reductase from *C. tenuis* and Man

parameter	yeast ALR[a]	human ALR[b]
$k_{cat,r}$ (s^{-1})[c]	17.0	0.20
$K_{m,RO}$ (mM)[c,d]	76	1.5
$k_{cat,r}/K_{m,RO}$ (M^{-1} s^{-1})	224	133
$k_{cat,o}$ (s^{-1})[c]	1.1	0.05
$K_{m,ROH}$ (mM)[c]	209	150
$k_{cat,o}/K_{m,ROH}$ (M^{-1} s^{-1})	5.26	0.33
$K_{i,NAD(P)^+}$ (μM)	195	0.006 (0.08)[g]
$K_{i,NAD(P)H}$ (μM)	16	0.010 (0.05)[g]
ratio: $K_{i,NAD(P)^+}/K_{i,NADPH}$	12.2	0.6 (1.6)[g]
ratio: $(k_{cat,r}/K_{m,RO})/(k_{cat,o}/K_{m,ROH})$	42.6	400
Haldane[e]	520	240 (640)
log fraction of K_{eq}' (%)[f]	60	≈100 (94)

[a] Determined at 25 °C, pH 7.0, in 50 mM phosphate buffer (adapted from ref *18*). [b] Determined at 25 °C, pH 8.0, in 33 mM phosphate buffer (adapted from ref *3*). [c] r, aldehyde reduction; o, alcohol oxidation; RO, D-xylose; ROH, xylitol. [d] Based on total D-xylose concentration. [e] Calculated from the experimental parameters for the Haldane relationship of an ordered bi-bi mechanism (*42*), and the apparent equilibrium constant, K_{eq}', contains the concentration of hydronium ions; reported K_{eq} values are 0.550×10^{10} M^{-1} and 0.575×10^{10} M^{-1} for NADH- and NADPH-dependent reduction, respectively (*21*). [f] Calculated as 100(log (k_{cat}/K_m ratio)/[log (k_{cat}/K_m ratio) + log (K_i ratio)]). [g] Data in parentheses are from ref *24* obtained at 15 °C in K-Mops buffer, pH 8.0.

relative to the corresponding complexes of human ALR, reflecting dissociation constants (K_i values) for NADH and NAD^+ that are 1600- and 32 500-fold higher than the corresponding dissociation constants of hALR for NADPH and $NADP^+$. In contrast to hAR that binds NH and N with nearly equal affinity, the dissociation constant of E−NH is much lower than that of E−N for yALR. With a $K_{i,NAD^+}/K_{i,NADH}$ ratio of 12.2, the ratio of catalytic efficiencies for yALR, $[(k_{cat,r}/K_{m,RO})/(k_{cat,o}/K_{m,ROH})]$, constitutes only a 60% log fraction of the equilibrium constant, compared to 94−100% for hALR (Table 5). By this criterion, yALR appears to be less highly evolved than hALR for an essentially unidirectional catalysis. (It is to our knowledge not clear whether the yALR-mediated reduction of D-xylose occurs at equilibrium or off equilibrium *in vivo*.) However, one has to consider the constraint of the NAD^+/NADH ratio of approximately 3−10 during microbial growth (*43*, *44*), especially under oxygen limitation as in during fermentation of D-xylose by yeast. The K_i ratio of yALR (Table 5) seems to be matched to this physiological situation, and a drastic decrease of this ratio is thus expected to result in stronger product inhibition of yALR by NAD^+. Interestingly, the dissociation constant for E−N, when N is $NADP^+$, is low for yALR (1.5 μM; *18*). The ternary E−NH−RO complex of hALR, compared to that of the yALR, is stabilized by an additional 2.3 kcal/mol, reflecting a 51-fold lower apparent K_m of the human enzyme for D-xylose. However, apparent 'tighter' binding of aldehyde in hALR is probably a consequence of the slow nucleotide release (*3*) rather than due to a real increase in binding affinity relative to yALR. The free energy barrier, shown by arrows in Figure 3, is very similar for both enzymes (≈14.5 kcal/mol, based on D-xylose without correction for the free aldehyde form). The major difference between yALR and hALR is that the energy for transition state stabilization is derived from interactions with both NH *and* RO in yALR (this work), but apparently with NH *only* in the case of hALR (*24*). Binding affinities for the product xylitol are weak and comparable for both enzymes. Hence, unlike hALR, yALR binds *both* thermodynamically favored products, ROH and NAD^+, weakly and makes a differential use of the intrinsic binding energy of RO and NH, relative to ROH and N. Differential binding of substrate(s) and product(s) as a principle in evolutionary maximizing the catalytic effectiveness of enzyme action has been described (*26*−*28*).

DISCUSSION

Steady-state kinetic studies for the NADH-dependent aldehyde reduction by ALR from the yeast *Candida tenuis* revealed significant differences to the mechanistically and structurally well-characterized human ALR that have hitherto not been pointed out. The differences pertain especially to the contribution of noncovalent enzyme−aldehyde interactions in the catalytic mechanism of the yeast enzyme, reflecting the different functions in physiology of hALR and yALR. The role of hydrogen-bonding interactions and hydrophobic bonding interactions with portions of the substrate other than the aldehyde group for transition state stabilization has to the best of our knowledge not been studied with any other ALR enzyme before. The results, in addition, are in good agreement with previous studies that used site-directed mutageneses (*2*, *16*) and molecular modeling (*22*) to identify interactions involved in the binding of the aldehyde substrate to the active site of hALR.

Structural Differences of Human and Yeast ALR and Implications for Catalysis. The ALRs from mammalian and microbial sources exhibit a 40% sequence identity (*5*), reflecting an overall structural similarity. The critical catalytic residues of human ALR (*2*, *45*, *46*) are strictly conserved in yALR (*5*, *6*, *13*), indicating that the chemical mechanism of catalysis is identical in both enzymes. However, compared to hALR (*15*), reactant binding is several orders of magnitude weaker as catalysis is faster with the ALR from yeast (Table 5). The different kinetic properties may be explained on the basis of (i) small differences in primary structure and (ii) a differential use of binding energy in catalysis of the two enzymes. In aldehyde reduction by human ALR, conformational changes in a nucleotide-clamping loop (residues Gly-213 to Leu-227; *7*) are required for the tight association with NADPH and, then, for release of $NADP^+$. The rate-limiting step is a conformational rearrangement of the ALR·$NADP^+$ binary complex prior to release of the oxidized nucleotide (*3*). A cysteine residue (Cys-298) stabilizes the closed conformation of the clamping loop (*16*). Oxidation or mutation of this cysteine residue in human ALR leads to a 9-fold increase in catalytic constants and a 40−50-fold increase of the apparent binding constants for NADPH and aldehyde (*15*). It is noteworthy that the increase in K_m for the aldehyde is actually due to an increase in the rate constant for the loop movement, that is, a reaction step not at all involved in aldehyde binding (*16*). In the ALRs from yeast, however, the residues of the clamping loop are poorly conserved, and the residue Cys-298 is not found at all (*5*, *6*, *13*), thus explaining weaker reactant binding and faster turnover (Table 5), relative to the human enzyme. The difference in catalytic efficiencies for nonpolar, aromatic aldehydes such as pyridine-3-carboxaldehyde for yeast and human ALR is more than 1300-fold, reflecting a 3400-fold higher K_m for the yeast enzyme. In addition to kinetic

argumentation (*3*), this result seems to reflect differences in the substrate binding pocket of hALR and yALR. Variations in the substrate binding pocket among the functionally diverse members of the aldo-keto reductase superfamily have been recently pointed out (*45, 46*).

Noncovalent Interactions: Hydrophobicity. Grimshaw (*24*) showed that for hALR the catalytic efficiencies for the reduction of various aldehydes are fairly constant, once correction is made for the free aldehyde present in solution. In addition, the values for k_{cat} are identical within statistical significance, reflecting the slow isomerization step of the enzyme·NADP complex in aldehyde reduction. In marked contrast, the values of k_{cat}/K_m for ALR from *C. tenuis* increase 253-fold across a series of aliphatic aldehydes of the form R-CHO, with R ranging in size from $-CH_2CH_3$ to $-(CH_2)_6CH_3$. Hence, unlike the human enzyme, yALR relies on specific (hydrophobic) interactions with the aldehyde for catalytic competence, in terms of k_{cat}/K_m reflecting the overall activation free energy. However, the contribution of 14 kJ/mol to the binding energy for R of $-(CH_2)_6CH_3$, relative to $-CH_2CH_3$, is not high when a value of 13 kJ/mol for the intrinsic binding energy of one alkyl group is taken into consideration (*29*). The 2.6-fold variation of k_{cat} in response to structural features of the substrate is, however, significant and indicates that, in contrast to hALR, the ALR from yeast can use binding energy derived from hydrophobic interactions with the aldehyde to reduce the activation energy for the rate-determining step, represented by k_{cat}.

Noncovalent Interactions: Hydrogen Bonding. In spite of its broad substrate specificity, the active site of hALR is not permissive and can discriminate 26-fold between the stereosisomers of polyhydroxylated aldehydes such as D-xylose and L-xylose (*2*). In terms of k_{cat}/K_m, a His-110 → Ala mutant enzyme displayed a reduced (4-fold) preference for D-xylose. Molecular modeling of aldehyde binding at the active site of hALR (*22*) suggested that the correctly oriented 2-OH group has hydrogen bonds with Hϵ1 of Trp-111, a residue not conserved in yeast ALRs, O7 of NADPH, and Nϵ2 of His-110.

To obtain estimates of the energetic contribution of hydrogen bonds in catalysis of yALR, we have used a complementary approach to site-directed mutagenesis that involves specific modifications of the aldehyde substrate rather than the enzyme. Deoxy and fluorodeoxy analogues of sugars such as D-galactose seem ideal for such studies because of the sterically conservative nature of the substitution (*47 ,48*). The variations in the observed values for k_{cat} and k_{cat}/K_m with deoxygenated substrate analogues, compared to the parent compound, are interpreted as a combination of electronic effects and differences in binding, particularly at the transition state.

(1) Electronic Effects. The transition state of the ALR-catalyzed aldehyde reduction is assumed to contain a partial negative charge on the substrate oxygen (*12*). Replacement of one hydroxyl of D-galactose by a more electronegative fluorine could arguably destabilize the transition state, thus slowing the reaction. The opposite situation is expected for deoxy analogues in which the substituent is hydrogen. Since this trend is not at all observed with yALR, noncovalent enzyme/substrate interactions with the hydroxyl group(s) of the aldehyde are apparently important for catalysis.

(2) Binding Effects. On the basis of binding effects alone, the deoxy and deoxyfluoro analogues should both be poorer substrates than the parent compound. However, because the fluorine substituent can accept, although not donate, hydrogen bonds whereas the hydrogen substituent cannot participate in either hydrogen-bonding interaction, the deoxy analogues should be worse than the deoxyfluoro, which was observed. For yALR, removal of all hydrogen-bonding interactions at C-2 in D-galactose results in a loss of binding energy ≥17 kJ/mol. In contrast, fluorine, in 2-fluoro-2-deoxy-D-galactose, can partially replace the hydroxyl group, suggesting that the fluorine, thus the original hydroxyl, acts as an acceptor of hydrogen bond(s). The failure of the fluorine to replace the hydroxyl at the 2 position completely suggests the presence of one hydrogen bond with an apparent strength of ≈4.4 kJ/mol in which the hydroxyl acts as the donor. The remaining approximately 13 kJ/mol of binding energy is distributed between hydrogen bonds in which the C-2(*R*) hydroxyl group of the substrate acts as acceptor. The contribution of hydrogen bonds to lower the activation energy of the rate-determining step in yeast ALR is demonstrated by the 15-fold decrease of k_{cat} for the reduction of the fluorodeoxy analogue, relative to D-galactose. This again points out the differential use of binding energy, derived from interaction with the aldehyde substrate, in catalysis of human and yeast ALR.

Unlike the 2 position, the substitution of the hydroxyls by hydrogen at other carbon atoms had no effect on the catalytic efficiency for aldehyde reduction. The ALR from *C. tenuis* seems to discriminate between stereoisomers of C-2(*R*) sugars primarily because of avoiding nonfavorable interactions with hydroxyls at positions ≥3 that are not oriented properly. For example, the 6.2 kJ/mol loss of binding energy observed for the reduction of D-ribose compared to that of its C-3 epimer, D-xylose, matches the incremental Gibbs energy of a (nonfavorable) transfer of a hydroxyl group from an aqueous to a nonpolar environment such as *n*-octanol.

Physiological Implications. The hALR has recently been described as an 'ideal aldehyde removease' (*24*) that (i), because of its low $K_{i\ NADPH}$, is saturated with cofactor at all times, thus creating a 'superreactive enzyme·NADPH complex', and (ii) reduces a broad range of substrates with similar and high catalytic efficiency. To achieve the latter, hALR does not rely on specific interactions with nonreacting portions of the aldehyde substrate. In many yeasts such as *C. tenuis*, the ALR-catalyzed reduction to xylitol is the initial step in the catabolism of D-xylose, and thus high efficiency in carrying the carbon flux rather than catalytic flexibility is required for the yALR. In agreement with this notion, the ALR from *C. tenuis* can now be classified according to its kinetic properties as a relatively *monospecific*, high-k_{cat} aldose reductase as opposed to hALR, the *broad specificity*, low-k_{cat} counterpart enzyme. In a series of polyhydroxylated aldehydes, the k_{cat} and k_{cat}/K_m values for reduction by yALR vary 4.4- and 80-fold, respectively (this work), whereas across the same substrate range these kinetic parameters are constant for hALR (*24*). Matched to the physiological role of yALR, the preferred substrates of this enzyme are D-xylose and L-arabinose (based on the free aldehyde form), and specificity is achieved primarily by hydrogen-bonding (≥17 kJ/mol) and to a lesser extent by hydrophobic bonding (≤14

kJ/mol) interactions of the yALR·NADH complex with the R portion of the R-CHO substrate. Unlike hALR, yALR-mediated catalysis is selective and *not* adapted for the reduction of structurally diverse and potentially toxic aldehydes. It is interesting that evolution of aldose reductase arrived at mechanistically and (probably) structurally conservative solutions that allow the enzyme to act as catalyst (i) in cell detoxification, like hALR, or (ii) in mainstream catabolism of pentose sugars, like yALR.

ACKNOWLEDGMENT

C. Leitner (Institute of Food Technology, BOKU, Vienna) is thanked for the preparation of the 2-keto-aldose substrates, and Dr. A. Stütz (Institute of Organic Chemistry, TU Graz) for the generous gift of 5-deoxy-D-xylose.

REFERENCES

1. Petrash, M., Tarle, I., Wilson, D. K., and Quiocho, F. A. (1994) *Diabetes 43*, 955−959.
2. Bohren, K. M., Grimshaw, C. E., Lai, C.-J., Harrison, D. H., Ringe, D., Petsko, G. A., and Gabbay, K. H. (1994) *Biochemistry 33*, 2021−2032.
3. Grimshaw, C. E., Bohren, K. M., Lai, C.-J., and Gabbay, K. H. (1995) *Biochemistry 34*, 14356−14365.
4. Bohren, K. M., Bullock, B., Wermuth, B., and Gabbay, K. H. (1989) *J. Biol. Chem. 264*, 9547−9551.
5. Hallborn, J., Walfridsson, M., Airaksinen, U., Ojamo, H., Hahn-Hägerdahl, B., Penttilä, M., and Keränen, S. (1991) *Bio/Technology 9*, 1090−1095.
6. Bolen, P. L., Hayman, G. T., and Shepherd, H. S. (1996) *Yeast 12*, 1367−1375.
7. Wilson, D. K., Bohren, K. M., Gabbay, K., and Quiocho, F. A. (1992) *Science 257*, 81−84.
8. Bohrani, D. W., Harter, T. M., and Petrash, J. M. (1992) *J. Biol. Chem. 267*, 24841−24847.
9. Rondeau, J.-M., Tete-Favier, F., Podjarny, A., Reymann, J.-M., Barth, P., Biellmann, J.-F., and Moras, D. (1992) *Nature 355*, 469−472.
10. Flynn, T. G., Shires, J., and Walton, D. J. (1975) *J. Biol. Chem. 250*, 2933−2940.
11. Tarle, I., Bohrani, D. W., Wilson, D. K., Quiocho, F. A., and Petrash, M. (1993) *J. Biol. Chem. 268*, 25687−25693.
12. Grimshaw, C. E., Bohren, K. M., Lai, C.-J., and Gabbay, K. H. (1995) *Biochemistry 34*, 14374−14384.
13. Yokoyama, S.-I., Kinoshita, Y., Suzuki, T., Kawai, K., Horitsu, H., and Takamizawa, K. (1995) *J. Ferment. Bioeng. 80*, 603−605.
14. Del Corso, A., Barsacchi, D., Gianessi, M., Tozzi, M. G., Camici, M., and Mura, U. (1989) *J. Biol. Chem. 264*, 17653−17655.
15. Grimshaw, C. E., Shabbaz, M., Jahangiri, G., Putney, C. G., McKercher, S. R., and Mathur, E. J. (1989) *Biochemistry 28*, 5343−5353.
16. Grimshaw, C. E., Bohren, K. M., Lai, C.-J., and Gabbay, K. H. (1995) *Biochemistry 34*, 14366−14373.
17. Petrash, J. M., Harter, T. M., Devine, C. S., Olins, P. O., Bhatnagar, A., Liu, S., and Srivastava, S. K. (1992) *J. Biol. Chem. 267*, 24833−24840.
18. Neuhauser, W., Haltrich, D., Kulbe, K. D., and Nidetzky, B. (1997) *Biochem. J., 326*, 683−692.
19. Grimshaw, C. E., Shabbaz, M., and Putney, C. G. (1990) *Biochemistry 29*, 9947−9955.
20. Kubiseski, T. J., Hyndman, D. J., Morjana, N. A., and Flynn, G. T. (1992) *J. Biol. Chem. 267*, 6510−6517.
21. Rizzi, M., Erlemann, P., Bui-Thanh, N.-A., and Dellweg, H.-W. (1988) *Appl. Microbiol. Biotechnol. 29*, 148−154.
22. de Winter, H. L., and von Itzstein, M. (1995) *Biochemistry 34*, 8299−8308.
23. Flynn, T. G. (1982) *Biochem. Pharmacol. 31*, 2705−2712.
24. Grimshaw, C. E. (1992) *Biochemistry 31*, 10139−10145.
25. Bedford, J. J., Bagnasco, S. M., Kador, P. F., Harris, H. W., Jr., and Burg, M. B. (1987) *J. Biol. Chem. 262*, 14255−14259.
26. Albery, W. J., and Knowles, J. R. (1976) *Biochemistry 15*, 5631−5640.
27. Burbaum, J. J., Raines, R. T., Albery, W. J., and Knowles, J. R. (1989) *Biochemistry 28*, 9293−9305.
28. Burbaum, J. J., and Knowles, J. R. (1989) *Biochemistry 28*, 9306−9317.
29. Fersht, A. R. (1985) *Enzyme Structure and Mechanism*, Freeman, New York.
30. Bruinenberg, P. M. (1986) *Antonie van Leeuwenhoek 52*, 411−429.
31. van Dijken, J. P., and Scheffers, W. A. (1986) *FEMS Microbiol. Rev. 32*, 199−224.
32. Singh, A., and Mishra, P. (1995) in *Microbial Pentose Utilization—Progress in Industrial Microbiology*, Vol. 33, pp 119−145, Elsevier, Amsterdam.
33. Kern, M., Haltrich, D., Nidetzky, B., and Kulbe, K. D. (1997) *FEMS Microbiol. Lett. 149*, 31−37.
34. Morjana, N. A., and Flynn, T. G. (1989) *J. Biol. Chem. 264*, 2906−2911.
35. Huwig, A., Danneel, H.-J., and Giffhorn, F. (1994) *J. Biotechnol. 32*, 309−315.
36. Tsuchiya, T. (1990) *Adv. Carbohydr. Chem. Biochem. 48*, 91−277.
37. Grimshaw, C. E. (1986) *Carbohydr. Res. 148*, 345−348.
38. Collins, P., and Ferrier, R. (1995) Monosaccharides, p 41, Wiley, New York.
39. Feather, M. S., Flynn, T. G., Munro, K. A., Kubiseski, T. J., and Walton, D. J. (1995) *Biochim. Biophys. Acta 1244*, 10−16.
40. Kotecha, J. A., Feather, M. S., Kubiseski, T. J., and Walton, D. J. (1996) *Carbohydr. Res. 289*, 77−89.
41. Vuorinen, T., and Serianni, A. S. (1990) *Carbohydr. Res. 207*, 185−210.
42. Segel, I. (1976) Enzyme Kinetics, Wiley, New York.
43. Führer, L., Kubicek, C. P., and Röhr, M. (1980) *Can. J. Microbiol. 26*, 405−408.
44. Bae, W., and Rittman, B. E. (1996) *Biotechnol. Bioeng. 49*, 690−699.
45. Bruce, N. C., Willey, D. L., Coulson, A. F., and Jeffery, J. (1994) *Biochem. J. 299*, 805−811.
46. Jez, J. M., Bennett, M. J., Schlegel, B. P., Lewis, M., and Penning, T. M. (1997) *Biochem. J. 326*, 625−636.
47. Street, I. P., Rupitz, K., and Withers, S. G. (1989) *Biochemistry 28*, 1581−1587.
48. Street, I. P., Armstrong, C. R., and Withers, S. G. (1986) *Biochemistry 25*, 6021−6027.

BI9717800