Substrate specificity of sheep liver sorbitol dehydrogenase

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The substrate specificity of sheep liver sorbitol dehydrogenase has been studied by steady-state kinetics over the range pH 7–10. Sorbitol dehydrogenase stereo-selectively catalyses the reversible NAD-linked oxidation of various polyols and other secondary alcohols into their corresponding ketones. The kinetic constants are given for various novel polyol substrates, including L-glucitol, L-mannitol, L-altritol, D-altritol, D-iditol and eight heptitols, as well as for many aliphatic and aromatic alcohols. The maximum velocities (k_{cat}) and the substrate specificity-constants (k_{cat}/K_m) are positively correlated with increasing pH. The enzyme-catalysed reactions occur by a compulsory ordered kinetic mechanism with the coenzyme as the first, or leading, substrate. With many substrates, the rate-limiting step for the overall reaction is the enzyme-NADH product dissociation. However, with several substrates there is a transition to a mechanism with partial rate-limitation at the ternary complex level, especially at

low pH. The kinetic data enable the elucidation of new empirical rules for the substrate specificity of sorbitol dehydrogenase. The specificity-constants for polyol oxidation vary as a function of substrate configuration with D-xylo > D-ribo > L-xylo > D-lyxo $\approx$ L-arabino > D-arabino > L-lyxo. Catalytic activity with a polyol or an aromatic substrate and various 1-deoxy derivatives thereof varies with $-\text{CH}_2\text{OH} > -\text{CH}_2\text{NH}_2 > -\text{CH}_2\text{OCH}_3 \approx -\text{CH}_3$. The presence of a hydroxyl group at each of the remaining chiral centres of a polyol, apart from the reactive C2, is also nonessential for productive ternary complex formation and catalysis. A predominantly nonpolar enzymic epitope appears to constitute an important structural determinant for the substrate specificity of sorbitol dehydrogenase. The existence of two distinct substrate binding regions in the enzyme active site, along with that of the catalytic zinc, is suggested to account for the lack of stereospecificity at C2 in some polyols.

INTRODUCTION

Polyols [$\text{C}_n\text{H}_2(\text{H}_2\text{O})_n$], or alditols, are acyclic polyfunctional alcohols which, formally, are derived from aldoses or ketoses by reduction of the carbonyl group [1,2]. The polyols are classified as tetrityls, pentitols, hexitols etc., and may on the basis of their physico-chemical properties be regarded as simple carbohydrates. Many polyols are widely distributed in plant and animal phyla, occurring in organisms ranging from bacteria to higher animals, and are especially abundant in algae, fungi and plants [1,2]. However, few polyols with $n > 6$ occur in nature and only a limited number of long-chain polyols have been synthesized to date [2,3]. Many polyols and derivatives thereof have found widespread applications. Sorbitol and xylitol, for instance, are for reasons of dental health and diabetes control commonly used as sugar substitutes in nutrition [4], and various polyol esters are useful emulsifying agents, explosives and pharmaceuticals [1,2].

Numerous polyol dehydrogenases (ketose reductases) with different substrate specificities have been characterized, including enzymes that catalyse the interconversion of xylitol/D-xylulose, D-mannitol/D-fructose and ribitol/D-ribulose [4]. The *in vivo* oxidation of sorbitol (D-glucitol or L-gulitol, Scheme 1) at C2 to D-fructose is catalysed by sorbitol dehydrogenase (SDH, EC 1.1.1.14) [5].

First recognized by Hers [6] as an additional pathway in glucose metabolism the polyol, or sorbitol, pathway catalyses the conversion of aldoses into their corresponding ketoses via polyols by first aldose reductase (EC 1.1.1.21) and, subsequently, SDH [7]. The pathway functions as a bypass to glycolysis and the pentose phosphate pathway, and the increased flux via the polyol

pathway in hyperglycaemia has been implicated in disease mechanisms [8–10]. Apart from its involvement in the production of fructose in seminal plasma and a possible function in coenzyme regulation [7], the physiological role of the polyol pathway in nondiabetics remains unclear.

SDH belongs to the medium-chain dehydrogenase/reductase enzyme family, which also includes several classes of alcohol dehydrogenases (ADH, EC 1.1.1.1) and numerous other proteins [4,11]. Sheep liver SDH is a homotetrameric zinc-enzyme of 38-kDa subunits, each of which contains one catalytic centre and

Scheme 1 The sorbitol molecule

The numbering of carbon atoms (superscript left) and absolute configurations (superscript right) are indicated

shares 25 % sequence identity with mammalian ADH [4,11–13]. SDH isoenzymes have not been discerned, and the sheep liver enzyme is homogeneous on electrophoresis [14] and upon ultracentrifugation [12]. On the basis of sequence alignment and the crystal structure of the related horse liver ADH, a model has been constructed for the sheep liver SDH protomer [15]. In the SDH-NAD-sorbitol ternary complex the C2-OH is poised for reaction at the catalytic zinc, while the adjacent C1-OH apparently interacts with one or more enzymic residues [14,15].

Due to the involvement of SDH in the etiology of diabetic complications, the enzyme has recently become a target for drug design (see [10,14] and references cited therein). The substrate specificity of SDH is a premise for prospective rational design of potent and specific enzyme inhibitors. As there is a lack of consensus in the literature regarding the substrate spectrum of SDH, the present study was undertaken. The substrate spectrum obtained is correlated with previously published data and is also discussed with respect to the putative metabolic role of this enzyme.

EXPERIMENTAL

Materials

All reagents were of analytical quality and, unless stated otherwise, supplied by Sigma, Fluka or Merck. Solutions were made in milli-Q water. Glassware was routinely rinsed with 6 M HNO_3 and Milli-Q water prior to use.

Sheep liver SDH was obtained as a lyophilisate from Boehringer, and enzyme stock solutions were prepared as described previously [16]. The dialysed enzyme preparation was homogeneous on starch gel electrophoresis and stable for several days at 4 °C. The concentration of active enzyme in solution was determined spectrophotometrically, as previously described [14,16]. The coenzymes were obtained from Sigma. Because of the instability of the coenzyme in acid media, NADH stock solutions were made in 10 mM sodium phosphate pH 7.4 or 10 mM glycine/NaOH pH 9.9. Coenzyme solutions were kept in the dark at 4 °C for no longer than the day of use.

Polyols which were unavailable from commercial sources – including the hexitols (meso)-allitol, L-glucitol (D-gulitol), L-mannitol, L-altritol (L-talitol), D-altritol (D-talitol) and D-iditol, all the heptitols investigated, 1,2,3-heptanetriol (70 % DL-threo, 30 % DL-erythro), DL-threo-1,2,3-octanetriol, DL-erythro-1,2,3-octanetriol, 1,2,7,8-octanetetrol, 6-deoxy-6-nitro-D-iditol, 6-deoxy-L-mannitol, 3-deoxysorbitol, 6-deoxysorbitol, 1,6-dideoxysorbitol, 1-deoxy-1-aminosorbitol, *N*-methyl-*N*-octanoyl-1-deoxy-1-amino-sorbitol and various nitro derivatives of heptitols – were synthesized (see [2,3,17–19] and references cited therein for preparation of polyols and derivatives). The compounds synthesized were, on the basis of ^{13}C NMR, found to be ≥ 98 % pure. The 3-butene-1,2-diols and 3-butene-2-ol were obtained from Fluka/Fisher Scientific and Aldrich, respectively. 1,2,10-decanetriol was purchased from Toronto Research Chemicals.

Kinetic measurements

Initial velocities (v) were determined spectrophotometrically by measuring the rate of change in absorbance at 340 nm for NAD reduction or NADH oxidation at 23.5 °C, using a Philips PU 8675 spectrophotometer connected to an ABB Goertz SE 120 recorder. Reactions were initiated by adding an aliquot of enzyme stock solution to a 3.0-ml reaction mixture in a 10-mm light path quartz cuvette. Measurements were made by varying the substrate concentration, generally over the range $5 K_m$ to $1/5 K_m$, with a constant coenzyme concentration of 1 mM (NAD) or 100 μM

(NADH) in either 50 mM sodium phosphate pH 7.4 or 50 mM glycine/NaOH pH 9.9. Initial velocity measurements were made in duplicate and plotted in terms of v vs. $1/[\text{substrate}]$, where e is the concentration of enzyme active sites (subunits) in the assay cuvette. The catalytic-centre activity, k_{cat} , which is defined as the number of substrate molecules transformed per catalytic centre per second, was obtained from the intercept with the ordinate axis. The relative standard deviations obtained for k_{cat} and K_m by linear regression of the double-reciprocal plots were ≤ 10 %.

Determination of equilibrium constants

The thermodynamic equilibrium constants (K_{eq}) for the interconversion of various SDH substrates were determined spectrophotometrically at 340 nm by recording the time-dependent changes in the NAD/NADH ratio using a Hewlett–Packard HP 8452A diode array spectrophotometer. For each substrate pair, the calculation of K_{eq} was based on three independent determinations in 50 mM sodium phosphate, pH 7.4, at 25 °C, in which the concentrations of NAD (100–1000 μM) or NADH (20–150 μM) and of substrate were varied. The relative standard deviations obtained for K_{eq} were ≤ 20 %.

RESULTS

Polyol substrates

Table 1 lists the maximum velocities (k_{cat}) and substrate specificity-constants (k_{cat}/K_m) for various polyol substrates of SDH. All polyol samples investigated, including all possible tetritols, pentitols and hexitols as well as all heptitols investigated except L-glycero-L-galacto-heptitol, were oxidized with concomitant reduction of NAD. However, D-threitol, D-arabitol, L-glucitol and D-glycero-L-allo-heptitol are practically inert, and the activity recorded with each *pro analysi* quality sample may reflect slight (≤ 2 %) contamination. The present K_m values for D-mannitol vary somewhat from those previously published [16], possibly due to previous contamination by sorbitol. However, the present sample, obtained by recrystallization of commercial D-mannitol, was found pure (≥ 99 %) by ^{13}C NMR scanning (25000 individual spectra in $^2\text{H}_2\text{O}$).

In Table 1, it is noticeable that k_{cat} and k_{cat}/K_m are positively correlated with increasing pH. The specificity-constants for the principal polyol substrates vary with xylitol > sorbitol $\approx$ L-iditol > L-threitol $\approx$ ribitol $\approx$ allitol $\approx$ D-glycero-D-glucio-heptitol. Although their k_{cat}/K_m values vary considerably, xylitol, ribitol, allitol, galactitol, D-mannitol, L-iditol and D-glycero-D-glucio-heptitol show, over the range pH 7–10, the same k_{cat} as sorbitol; this also applies for L-threitol and D-glycero-D-allo-heptitol at high pH.

SDH is specific for reaction at C2 of a polyol substrate [4,5,12]. Hence, glycerol is oxidized to dihydroxyacetone [20]. The oxidation products of ribitol, xylitol, L-arabitol, allitol, D-glucitol (sorbitol or L-gulitol), L-iditol and galactitol have previously been identified as D-ribulose [21], D-xylulose [21], L-ribulose [12], D-psicose (D-allulose) [21], D-fructose [5,12], L-sorbose [12] and D-tagatose [22], respectively. Whereas the SDH-catalysed oxidation of L-threitol gives L-erythrulose, erythritol yields D-erythrulose. The respective products of oxidation of D- and L-mannitol and D- and L-altritol are considered to be D- and L-fructose and D- and L-psicose. D-Iditol can be expected to give D-sorbose. The enzyme-catalysed oxidations of D-glycero-L-alto-, D-glycero-D-gulo-, D-glycero-D-allo-, D-glycero-D-glucio-/D-glycero-D-manno-, D-glycero-L-galacto-, and D-glycero-L-gulo-heptitol can be predicted to yield D-talo-hept-2-ulose, D-

Table 1 Kinetic constants for polyol substrates of sorbitol dehydrogenase

Measurements were performed with a constant NAD concentration of 1 mM in 50 mM sodium phosphate pH 7.4 or 50 mM glycine/NaOH pH 9.9. n.d., not determined. TC, Theorell–Chance mechanism (i.e. the same k_{cat} as sorbitol, see main text).

Substrate	pH 7.4			pH 9.9		
	k_{cat} (s ⁻¹)	K_{m} (mM)	$k_{\text{cat}}/K_{\text{m}}$ (s ⁻¹ ·M ⁻¹)	k_{cat} (s ⁻¹)	K_{m} (mM)	$k_{\text{cat}}/K_{\text{m}}$ (s ⁻¹ ·M ⁻¹)
Glycol	0.25	6000	0.042	2.5	3800	0.66
Glycerol [20]	0.13	1450	0.090	0.63	300	2.1
(meso)-erythritol [14]	0.25	160	1.6	1.5	108	14
L-threitol [14]	1.8	15	120	7.3 [TC]	4.4	1659
D-threitol [14]	0.025	1400	0.018	0.25	760	0.33
(meso)-ribitol [16]	5.0 [TC]	51	98	7.3 [TC]	5.6	1304
(meso)-xylitol [16]	4.5 [TC]	0.90	5000	7.3 [TC]	0.70	10 400
L-arabitol	0.50	86	5.8	1.7	14	121
D-arabitol	0.033	260	0.13	0.050	190	0.26
(meso)-allitol	4.3 [TC]	42	102	7.0 [TC]	4.2	1667
meso-galactitol [16]	5.0 [TC]	680	7.4	7.3 [TC]	285	26
L-glucitol	0.13	300	0.43	0.25	167	1.5
D-glucitol [16]	5.0 [TC]	3.6	1389	8.3 [TC]	1.8	4611
(sorbitol)						
L-mannitol	0.35	750	0.47	1.3	390	3.3
D-mannitol	5.0 [TC]	600	8.3	7.5 [TC]	270	28
L-altritol	0.25	34	7.4	1.4	15	93
D-altritol	0.25	137	1.8	0.83	51	16
L-iditol [16]	5.0 [TC]	3.3	1516	9.0 [TC]	2.5	3600
D-iditol	0.33	9.0	37	1.3	7.8	167
Heptitols						
(meso)-D-glycero-L-altro-	0.065	8.8	7.4	2.1	65	32
(meso)-D-glycero-D-gulo-	0.030	23	1.3	0.50	188	2.7
(meso)-D-glycero-D-allo-	0.75	13	58	7.5 [TC]	11	682
D-glycero-D-gluco-	4.5 [TC]	25	180	8.0 [TC]	5.4	1481
(β-sedoheptitol)						
D-glycero-D-manno-	1.3	80	16	2.8	30	93
(volemitol)						
D-glycero-L-galacto-	0.18	49	3.7	1.3	102	13
D-glycero-L-allo-	0.10	82	1.2	0.20	45	4.4
D-glycero-L-gulo-	0.21	20	11	1.4	57	25

gluco-hept-2-ulose, D-allo-hept-2-ulose, D-altro-hept-2-ulose, D-glycero-L-lyxo-hept-2-ulose and D-ido-hept-2-ulose, respectively. The keto-hexose L-sorbose is oxidized to 5-keto-D-fructose with, at pH 9.9, $k_{\text{cat}} = 0.83 \text{ s}^{-1}$ and $K_{\text{m}} = 240 \text{ mM}$. D-fructose is oxidized to 5-keto-D-fructose with $k_{\text{cat}} = 0.25 \text{ s}^{-1}$ and $K_{\text{m}} = 825 \text{ mM}$ at pH 9.9. Primary aliphatic alcohols, including ethanol, 1-propanol and pantothenyl alcohol, are unreactive, as are lactate, cyclic polyfunctional alcohols (myoinositol) and methyl β-D-glucopyranoside.

Deoxy polyol substrates

The kinetic constants for various deoxy polyol substrates are given in Table 2. Data for 6-deoxy-6-nitro-D-iditol were collected only at pH 7.4 due to the instability of this compound in alkaline solution. The kinetic constants for 1-deoxy-1-thiosorbitol were determined only at pH 7.4 in order to avoid interferences from adduct formation with the coenzyme at high pH [14,23]. The NAD-linked oxidation of each of the alkylidiols and alkyltrials occurs stereospecifically with the enantiomer with the configuration (2*R*) corresponding to the 2*S*-configuration of sorbitol. For the various alkylidiols and alkyltrials, $k_{\text{cat}}/K_{\text{m}}$ is positively correlated with increasing carbon chain length up to C₇. As with the polyols, k_{cat} and $k_{\text{cat}}/K_{\text{m}}$ increase toward high pH, and (*R*)-

1,2,4-butanetriol, (*R*)-1,2-pentanediol, (*R*)-1,2-hexanediol and 1-deoxy-1-aminosorbitol show the same k_{cat} as sorbitol at pH 9.9. Good substrates are also DL-*threo*-1,2,3-octanetriol, 1,2,3-heptanetriol, 3-deoxysorbitol and 6-deoxysorbitol. Whereas the 6-deoxy analogues of sorbitol and L-mannitol exhibit specificity-constants 2–3 times higher than those of their respective parent molecules (Table 1), the 1-deoxy analogues show much lower $k_{\text{cat}}/K_{\text{m}}$ values which vary with $-\text{CH}_2\text{NH}_2 > -\text{CH}_3$.

Unlike the other bifunctional butanols, 1,4-butanediol is as expected not oxidized. Neither 1-deoxy-1-nitro-D-glycero-D-galacto-heptitol nor the 7-deoxy-7-nitro derivatives of various heptitols, including D-glycero-L-gulo-, D-glycero-L-allo-, D-glycero-D-manno-, D-glycero-L-galacto- and L-glycero-L-galacto-heptitol, are substrates. *N*-methyl-*N*-octanoyl-1-deoxy-1-aminosorbitol, 3-butene-2-ol and 1,5-anhydrosorbitol are also inert.

Aromatic substrates

Table 3 lists the kinetic constants for various aromatic substrates. Each of the oxidative reactions occurs stereospecifically with the enantiomer having the configuration (generally 2*R*) corresponding to the 2*S*-configuration of sorbitol. The k_{cat} and $k_{\text{cat}}/K_{\text{m}}$ values are positively correlated with increasing pH. (*R*)-1-phenyl-1,2-ethanediol (phenylglycol) is oxidized to (*R*)-1-phenyl-2-

Table 2 Kinetic constants for deoxy polyol substrates of sorbitol dehydrogenase

Measurements were performed with a constant NAD concentration of 1 mM in 50 mM sodium phosphate pH 7.4 or 50 mM glycine/NaOH pH 9.9. n.d., not determined. TC, Theorell–Chance mechanism (i.e. the same k_{cat} as sorbitol, see main text). * Mixture of D-, L- and *meso* forms. § 70% DL-*threo*, 30% DL-*erythro*.

Substrate	pH 7.4			pH 9.9		
	k_{cat} (s ⁻¹)	K_m (mM)	k_{cat}/K_m (s ⁻¹ M ⁻¹)	k_{cat} (s ⁻¹)	K_m (mM)	k_{cat}/K_m (s ⁻¹ M ⁻¹)
(<i>R</i>)-1,2-propanediol [20]	0.20	825	0.24	1.3	125	10
(<i>R</i>)-1,2-butanediol	0.21	36	5.8	2.5	42	60
2,3-butanediol*	0.50	90	5.6	1.5	113	13
(<i>S</i>)-2,4-butanediol		n.d.		0.063	70	0.90
(<i>R</i>)-1,2,4-butanetriol	1.4	14	100	7.5 [TC]	26	288
(2 <i>S</i> , 3 <i>S</i>)-1,4-dimethoxy-2,3-butanediol		n.d.		0.50	85	5.9
(2 <i>R</i>)-3-butene-1,2-diol	0.30	22	14	2.8	14	200
(<i>R</i>)-1,2-pentanediol	0.75	6.5	115	7.3 [TC]	13	562
(<i>R</i>)-1,2-hexanediol	1.0	2.4	417	7.5 [TC]	4.0	1880
1,2,3-hexanetriol*	0.075	16	4.7	0.63	12	53
(<i>R</i>)-1,2,6-hexanetriol	0.88	10	88	4.3	7.5	573
1,2,3-heptanetriol§		n.d.		1.7	0.38	4474
DL- <i>threo</i> -1,2,3-octanetriol	1.7	4.9	347	4.3	1.6	2688
DL- <i>erythro</i> -1,2,3-octanetriol	0.20	23	8.7	1.7	26	65
1,2,7,8-octanetetrol*		n.d.		0.50	40	13
(<i>R</i>)-1,2,10-decanetriol	0.063	2.2	29	0.55	3.7	149
6-deoxy-6-nitro-D-iditol	0.28	184	1.5		n.d.	
6-deoxy-L-mannitol (L-rhamnitol)		n.d.		0.040	3.8	11
1-deoxy-1-thio-sorbitol [14]	0.043	8.6	5.0		n.d.	
1-deoxy-1-amino-sorbitol	1.3	58	22	8.3 [TC]	3.3	2515
3-deoxysorbitol	4.5 [TC]	7.5	600	7.8 [TC]	6.9	1130
6-deoxysorbitol	3.1	1.1	2818	6.3	0.70	9000
1,6-di-deoxysorbitol	1.0	15	67	5.0	20	250

Table 3 Kinetic constants for aromatic substrates of sorbitol dehydrogenase

Measurements were performed with a constant NAD concentration of 1 mM in 50 mM sodium phosphate pH 7.4 or 50 mM glycine/NaOH pH 9.9. n.d., not determined. TC, Theorell–Chance mechanism (i.e. the same k_{cat} as sorbitol, see main text).

Substrate	pH 7.4			pH 9.9		
	k_{cat} (s ⁻¹)	K_m (mM)	k_{cat}/K_m (s ⁻¹ · M ⁻¹)	k_{cat} (s ⁻¹)	K_m (mM)	k_{cat}/K_m (s ⁻¹ · M ⁻¹)
(<i>R</i>)-1-phenyl-1,2-ethanediol [14]	1.7	3.5	486	7.0 [TC]	1.0	7000
(<i>S</i>)-1-phenylethanol		n.d.		0.10	7.0	14
(<i>R</i>)-1-phenyl-2-aminoethanol	0.13	40	3.3	2.0	1.5	1333
(<i>R</i>)-1-(<i>p</i> -hydroxy)-phenyl-2-aminoethanol (octopamine)	0.13	1.3	100	7.5 [TC]	15	500
(<i>S</i>)-1- <i>O</i> -benzylglycerol		n.d.		0.63	2.7	233

hydroxyethanone (ω -hydroxyacetophenone) with, at high pH, kinetic constants similar to those for sorbitol. Figure 1 shows the pH dependence of the k_{cat} ratio for (*R*)-phenylglycol and sorbitol over the range pH 5–10. The k_{cat} and k_{cat}/K_m (not shown) ratios are similarly affected by pH, with pK dependencies of 8.6 and 9.0, respectively. As for the deoxy polyols, (*R*)-phenylglycol and its 1-deoxy analogues (*S*)-1-phenylethanol and (*R*)-1-phenyl-2-aminoethanol are oxidized with specificity-constants that vary

Figure 1 The pH dependence of the k_{cat} ratio for (*R*)-1-phenyl-1,2-ethanediol (PG) and sorbitol (S)

The kinetic constants were determined with a constant [NAD] = 1 mM in 50 mM sodium phosphate pH $\leq$ 8.4 or 50 mM glycine/NaOH pH $\geq$ 8.8.

with $-\text{CH}_2\text{OH} > -\text{CH}_2\text{NH}_2 > -\text{CH}_3$. The biogenic amine 1-(*p*-hydroxyphenyl)-2-aminoethanol (octopamine) is oxidized to the corresponding ketone with, at high pH, the same k_{cat} as for

Table 4 The pH independent equilibrium constants (K_{eq}) for the interconversion of sorbitol dehydrogenase substrates

$K_{eq} = [\text{ketone}][\text{NADH}][\text{H}^+]/[\text{alcohol}][\text{NAD}^+]$ was determined at 25 °C in 50 mM sodium phosphate, pH 7.4. Mean values ($\pm$ S.D.) based on three separate experiments are given.

Substrate pair	$K_{eq} \times 10^{12}$ (M)
glycerol/dihydroxyacetone	1.1 ($\pm$ 0.2)
L-threitol/L-erythrose	6.5 ($\pm$ 0.5)
xylitol/D-xylulose	63 ($\pm$ 6)
ribitol/D-ribulose [25]	330 ($\pm$ 120)
sorbitol/D-fructose [26]	3700 ($\pm$ 300)
galactitol/D-tagatose [22]	1300*
D-glycero-D-gluco-heptitol/D-altro-hept-2-ulose	340 ($\pm$ 40)
1-phenyl-1,2-ethanediol/1-phenyl-2-hydroxyethanone	140 ($\pm$ 15)
1-(<i>p</i> -hydroxyphenyl)-2-aminoethanol/1-(<i>p</i> -hydroxyphenyl)-2-aminoethanone	160 ($\pm$ 20)
1-phenyl-2-aminoethanol/1-phenyl-2-aminoethanone	6.5 ($\pm$ 1)
2-phenyl-2-hydroxyacetaldehyde/2-phenyl-2-oxoacetaldehyde	12 000 ($\pm$ 2000)

*Error bars not available.

sorbitol. The kinetic parameters for the reduction of 2-phenyl-2-oxoacetaldehyde (phenylglyoxal), determined with 100 μ M NADH in 50 mM sodium phosphate pH 7.4, are $k_{cat} = 13 \text{ s}^{-1}$ and $K_m = 88 \text{ mM}$. The product of phenylglyoxal reduction is (*R*)-2-phenyl-2-hydroxy-acetaldehyde (phenylglycol aldehyde). Mandelic acid, paracetamol, phenacetin, 2-phenylethanol, 1-phenylmethanol and 1,1-diphenylmethanol are not substrates. Various related compounds, including adrenaline, noradrenaline [24], (2*R*, 3*R*)- and (2*S*, 3*S*)-*trans*-3-phenyloxiran-2-methanol, are also inert.

Equilibrium constants

Table 4 lists the pH independent thermodynamic equilibrium constants (K_{eq}) for the interconversion of various SDH substrates. Here, $K_{eq} = [\text{ketone}][\text{NADH}][\text{H}^+]/[\text{alcohol}][\text{NAD}]$, and the pH dependent equilibrium constant $K'_{eq} = K_{eq}/[\text{H}^+]$. Due to the liberation of a proton in each of the oxidative reactions, K'_{eq} is positively correlated with increasing pH.

DISCUSSION

Kinetic mechanism

Sorbitol oxidation and fructose reduction catalysed by sheep liver SDH follow a compulsory ordered (Theorell–Chance) kinetic mechanism (I) with NAD(H) as the first, or leading, substrate and the enzyme–coenzyme product dissociation (k_{-1} or k'_{-1}) as the rate-determining step for the overall reaction [16,26]:

where E = SDH, O = NAD, R = NADH, S = sorbitol and F = fructose. Hence, a substrate independent maximum velocity is inherent in the mechanism, and the corresponding maximum velocities for oxidation ($k_{cat} = 1/k'_{-1}$) of sorbitol and various other polyol substrates imply a similar mechanism and a common rate-limiting enzyme–NADH product dissociation. Similar maximum velocities with various polyol substrates have previously been reported also for human [27] and rat [28] liver SDH, as well as for the human [29], rat [30] and bovine [31] brain enzymes, suggesting a compulsory ordered mechanism, without kinetically

significant ternary complexes, for the various mammalian enzymes.

Xylitol (2*S*,3*R*,4*R*) and its 3-epimer ribitol, sorbitol (2*S*,3*R*,4*R*,5*R*) and each of its four epimeric isomers and D-glycero-D-gluco-heptitol (2*S*,3*R*,4*R*,5*R*,6*R*) conform to mechanism (I) over the range pH 7–10. The enzyme-catalysed oxidation of L-threitol (2*S*,3*S*), D-glycero-D-allo-heptitol (2*S*,3*S*,4*R*,5*R*,6*R*), (2*R*)-1,2,4-butanetriol, (2*R*)-1,2-pentanediol, (2*R*)-1,2-hexanediol, 1-deoxy-1-aminosorbitol, (*R*)-octopamine and (*R*)-phenylglycol, all of which have the configuration of sorbitol at C2, conform to (I) only at high pH. For the substrates that deviate from the Theorell–Chance mechanism, rate-limitation for the overall reaction is imposed by kinetic or catalytic steps other than the enzyme–NADH product dissociation *per se* [20]. The observation that the decrease in the k_{cat} ratio (Figure 1) for (*R*)-phenylglycol and sorbitol toward low pH parallels that in the k_{cat}/K_m ratio, and the particularly low k_{cat}/K_m values for substrates not conforming to (I), indicate that ternary complexes become involved in rate-determination at low pH [20]. This is supported by isotope effects on k_{cat}/K_m with ($^2\text{H}_8$)-sorbitol, which indicate that ternary complexes with deuterated sorbitol become kinetically significant at low substrate concentrations, especially toward low pH [16,26]. Similar isotope effects and deviations from the Theorell–Chance mechanism have previously been reported for the related ADH [32,33].

Substrate specificity of mammalian sorbitol dehydrogenase

There has for many years been lack of consensus in the literature regarding the substrate specificity of SDH. A previously formulated rule states that the polyol substrates of SDH have the D-*cis*-2,4-dihydroxy (2*S*,4*R*) configuration (e.g. sorbitol) and a primary hydroxyl group adjacent to the oxidation site at C2 [1,12,21]. The present study shows that sheep liver SDH catalyses the reversible NAD-linked oxidation of most polyols – including D-mannitol and galactitol, the 2- and 4-epimers of sorbitol, respectively – into their corresponding ketoses. Numerous reports corroborate that D-mannitol [12,27–30,34–38] and galactitol [12,27,29–31,38], as well as L-arabitol [12,27,29,30,38], are substrates for SDH. Hence, the putative D-*cis*-2,4-dihydroxy requirement for the polyol substrates of SDH is not absolute. The substrate spectrum obtained should also apply for other mammalian sorbitol dehydrogenases, which show 80–90 % sequence identity [39].

On the basis of their configuration at C2–C4, the pentitols, hexitols and heptitols constitute eight distinct groups (Figure 2). As the numbering of carbon atoms in polyols can be from either end of the chain, each substrate is categorized on the basis of the most favourable configuration (i.e. in relation to sorbitol). The L-ribo configuration is redundant, because all polyols that may be ascribed to this group also have a more favourable configuration upon numbering the chain from the opposite terminus. The seven remaining configurations are: (1) D-xylitol (D-glucitol, L-iditol, D-glycero-D-gluco-heptitol); (2) L-xylitol (D-iditol); (3) D-ribo (ribitol, allitol, L-altritol, D-glycero-D-allo-heptitol); (4) D-lyxose (D-arabitol, D-mannitol, D-glycero-D-manno-heptitol, D-glycero-L-gulo-heptitol); (5) L-lyxose (L-glucitol, L-mannitol, D-glycero-D-gulo-heptitol); (6) L-arabino (L-arabitol, galactitol, D-glycero-L-altro-heptitol); (7) D-arabino (D-altritol, D-glycero-L-galacto-heptitol, D-glycero-L-allo-heptitol). SDH shows low specificity with respect to the configuration at C5, and all heptitols investigated have the same configuration at C6. Hence, for correlating polyol configuration and activity the aforementioned categories suffice. For the hexitols and heptitols, the substrate specificity-constants vary with D-xylitol > D-ribo > L-xylitol > D-lyxose $\approx$ L-arabino > D-arabino > L-lyxose. The finding

Figure 2 The configuration of pentitols, hexitols and heptitols at C2–C4

The L-ribo configuration is redundant, because all polyols that may be ascribed to this group also possess a more favourable configuration upon numbering the chain from the opposite terminus (see main text).

that L-glycero-L-galacto-heptitol, which has the L-lyxo configuration but opposite chirality at C5 in relation to D-glycero-D-gulo-heptitol, is not a substrate that accords with this scheme. For the pentitol substrates, the specificity-constants vary with D-xylo > D-ribo > L-arabino. In agreement with the specificity for oxidation of the C₅–C₇ polyols, k_{cat}/K_m for the tetrutols varies with L-threitol > erythritol.

The results indicate a functionally important, although not essential, role for the primary hydroxyl group at C1. This is corroborated by the previous observations that the 1-deoxy derivatives of sorbitol, D-mannitol and D-fructose are substrates for sheep liver SDH [36]. The present study shows that a $-\text{CH}_2\text{NH}_2$, $-\text{CH}_2\text{SH}$, $-\text{CHO}$ or $-\text{CH}_2\text{OCH}_3$ group adjacent to C2 is also possible. However, species containing a carboxylate group or a large substituent adjacent to C2 are not oxidized. While the aldehyde group may be hydrated in the enzyme-NAD(H)-substrate complexes, k_{cat}/K_m for an unsubstituted polyol substrate and corresponding 1-deoxy derivatives varies with $-\text{CH}_2\text{OH} > -\text{CH}_2\text{NH}_2 > -\text{CH}_2\text{OCH}_3 \approx -\text{CH}_3$. This parallels the different hydrogen bonding capabilities of the substituents and, thus interpreted, the pronounced pH dependence of k_{cat}/K_m for 1-deoxy-1-aminosorbitol reflects the poor hydrogen bonding capability of the amino group at low pH. The apparently low specificity-constant for 1-deoxy-1-thiosorbitol probably reflects a depression of the intrinsic value, due to thiolate binding to zinc inhibiting the oxidative reaction [14]. The correlation between k_{cat}/K_m and the hydrogen bonding capability of the C1 substituent of a substrate accords with that seen previously with various reversible inhibitors [14]. The results accord with the concept that, while the reactive C2-OH of a substrate is poised for reaction at the catalytic zinc in an enzyme-NAD-substrate ternary complex, the C1-OH forms a specific hydrogen bond with enzymic residues which, according to the SDH structure model, are Glu154/Lys293 [15]. This is supported by site-directed mutagenesis studies on the rat liver enzyme [41], and by the fact that *Bombyx mori* SDH, which has a Gln residue in the position equivalent to Glu154 in the sheep liver enzyme, shows an extremely low affinity for sorbitol [42].

The results indicate a nonessential role for the C3-OH, consistent with the previous observations that 3-deoxyxylylitol and

3-deoxy-D-fructose are good substrates [43,44]. The C4-OH is also nonessential, although a dihydroxy arrangement is important *per se* and for the butanediols k_{cat}/K_m varies with $1,2 \approx 2,3 > 1,3$ -butanediol. At C5 (D-xylo) the ketohexose L-sorbose is oxidized to 5-keto-D-fructose with a specificity-constant which is 0.1 % of that for L-iditol. However, when correction is made for the fact that L-sorbose is $\geq 99.9\%$ cyclized [45], the hexose is a good substrate with a specificity-constant similar to that of L-iditol. This indicates a nonessential role also for the C5-OH. D-fructose is also oxidized at C5 (D-lyxo) to 5-keto-D-fructose with a specificity-constant which is 0.01 % of that for sorbitol, consistent with D-xylo > D-lyxo. The data for the 6-deoxy analogues of L-mannitol, sorbitol and (2R)-1,2,6-hexanetriol show that the C6-OH is not only nonessential, but that a methyl group in this position is actually favourable. However, a $-\text{NO}_2$ group at C6 or C7 is highly unfavourable.

Aromatic secondary alcohols have previously been reported to be substrates for mammalian liver SDH [14,24]. The high k_{cat}/K_m ratios for (R)-phenylglycol/glycol and phenylglyoxal/methylglyoxal [20], and the pronounced increase in k_{cat}/K_m upon lengthening the carbon chain of the alkyldiols and alkyltrials up to C7, suggest that the deoxy polyols and aromatics interact with an enzymic nonpolar epitope in the enzyme-NAD(H)-substrate complexes. The existence of a hydrophobic site in the vicinity of the catalytic metal complex in SDH is indicated by the enzyme structure model [15], and nonpolar interactions appear involved also in the binding of reversible inhibitors [14] and affinity labelling reagents [46,47]. Inspection of the three-dimensional conformations of the polyols investigated [2,18,19] raises the possibility that the nonpolar backbone of various polyols is involved in hydrophobic interactions with the enzyme. For the polyols with the favourable *cis*-2,4 dihydroxy configuration (D-xylo, L-xylo, D-ribo; Figure 2), an intramolecular 2,4 parallel O/...O interaction induces bending of the carbon chain at C2, stabilizing the energetically most stable (bent chain) conformation with a nonpolar backbone. However, with the D-ribo configuration, the C3-OH disturbs the 2,4 interaction and destabilizes the nonpolar backbone of the substrate. In the absence of any 2,4 interaction (D-lyxo, L-lyxo, D-arabino, L-arabino), an extended zig-zag conformation without a nonpolar

Figure 3 Representation of polyol conformations

(a) Bent chain conformation with a nonpolar backbone induced by the *cis*-2,4-dihydroxy configuration; (b) extended zig-zag conformation without a nonpolar backbone. Carbon, hydrogen and oxygen atoms are black, white and hatched, respectively.

backbone results. Figure 3 depicts the conformations of two pentitols having either the bent chain conformation with a nonpolar backbone induced by the *cis*-2,4-dihydroxy configuration (a), or an extended zig-zag conformation without a nonpolar backbone (b). Thus interpreted, the presence of hydroxyl groups, apart from the reactive C2-OH and to a lesser extent the C1-OH, is nonessential for binding and catalytic activity. Indeed, the lack of hydroxyl groups appears favourable in the case of an intramolecular polar interaction destabilizing the nonpolar backbone of a substrate. These structural considerations accord with the aforementioned empirical generalizations regarding the specificity of SDH toward polyols.

The epimeric ratios of k_{cat}/K_m for sorbitol/D-mannitol and D-glycero-D-gluco-heptitol/D-glycero-D-manno-heptitol are 170 and 11, respectively, at pH 7.4. These results show that, unlike the situation with alkylalcohols and aromatics, SDH does not exhibit absolute substrate stereoselectivity with respect to the configuration at C2 of a polyol, and that the degree of enzymic discrimination between 2-epimers is positively correlated with decreasing carbon chain length. The situation with similar k_{cat} values, but very different specificity-constants, for the oxidation of 2-epimers parallels that seen previously with ADH and various epimeric secondary alcohols [48,49]. In the case of ADH, the lack of absolute substrate stereoselectivity has been attributed to either the existence of two distinct alkyl binding regions in the enzyme active site [49], or a single alkyl binding region combined with a capability of the active site to accommodate an unbound alkyl group [48]. Liver ADH oxidizes both enantiomers of various secondary alcohols, as the alkyl groups can be in either of two hydrophobic regions in the active site [48]. However, in the case of yeast ADH one hydrophobic region only accepts alkyl groups smaller than propyl, and this enzyme only oxidizes secondary alcohols containing at least one methyl or ethyl group [48]. This steric discrimination by yeast ADH implies that when

the second alkyl group is larger than ethyl, the reaction shows absolute stereoselectivity. The structural determinants for the specificity of SDH appear to include a polar region and a predominantly hydrophobic region. Figure 4 depicts chiral interactions in the SDH active site similar to those for the binding of secondary alcohols to ADH. While the C2-OH is co-ordinated to the catalytic zinc atom and the C2-H poised for *pro-R* specific hydride transfer to NAD, the phenyl, alkyl or polyhydroxy substituent (R) of a substrate is in a predominantly hydrophobic region and the $-\text{CH}_2\text{OH}$ moiety occupies a polar area in the active site. While the polar site may accommodate a substituent as large as $-(\text{CHOH})_4-\text{CH}_2\text{OH}$, steric effects inhibit binding of substituents larger than a phenyl or $-(\text{CH}_2)_4-\text{CH}_3$ group to the hydrophobic site. In the case of R being a non-polar group, an inversion in relation to sorbitol of the configuration at C2 results in a most unfavourable arrangement, in agreement with the strict stereoselectivity for the oxidation of alkylalcohols and aromatics. However, a similar inversion at C2 in a polyol places the polyhydroxy group in the polar area while the $-\text{CH}_2\text{OH}$ substituent occupies the predominantly hydrophobic site. A corollary is that substitution of the $-\text{CH}_2\text{OH}$ moiety at C2 with a nonpolar group should have opposite effects on the k_{cat}/K_m of epimeric substrates. Indeed, whereas various 1-deoxy analogues of sorbitol are comparatively poor substrates, 1-deoxy-D-mannitol has been reported to show a specificity-constant forty times higher than that of D-mannitol [36].

Metabolic role of sorbitol dehydrogenase

On the basis of substrate specificity studies, aldose reductase has been ascribed activities in detoxification [50,51]. The fact that polyols have few metabolic functions in mammalian tissues [7] and the differential tissue distributions [52,53] of aldose reductase

Figure 4 Schematic representation of putative chiral interactions of polyols (2S) and the equivalent (priority difference) alkyl- and aromatic alcohol substrates with the SDH active site

R denotes a phenyl, alkyl or polyhydroxy group of a substrate. SDH catalyses nucleophilic attack by the C2 hydrogen of an alcohol substrate on the *re* side of NAD⁺. (a) indicates hydrogen bonding with the enzyme, and (b) denotes a hydrophobic epitope. With the (2*R*)-polyols having substrate activity, the polyhydroxy and hydroxymethyl substituents are inverted to fit in with the stereospecificity for hydride transfer.

and SDH also question the physiological significance of the polyol pathway in normal tissues. It is interesting, that 1-phenylethanol, phenylglycol and phenylglyoxal are substrates for SDH, because these species are intermediates in the hepatic catabolism of ethylbenzene and styrene [24,54]. The former is metabolized via the sequence ethylbenzene → 1-phenylethanol → acetophenone → ω -hydroxyacetophenone → (*R*)-phenylglycol/phenylglyoxal → (*R*)-phenylglycol aldehyde → (*R*)-mandelic acid, while styrene is metabolized via phenylglycol to racemic mandelic acid. SDH may control the stereospecific conversions of (*S*)-1-phenylethanol to acetophenone, ω -hydroxyacetophenone to (*R*)-phenylglycol, and phenylglyoxal to (*R*)-phenylglycol aldehyde. While the epoxides of styrene and phenylglycerol are not substrates, they are sorbitol-competitive inhibitors with, at pH 7.4, K_i ($K_{\text{enzyme-NAD-epoxide}}$) values of 9.0 mM, 0.48 mM and 0.69 mM for styrene oxide, (2*R*,3*R*)-*trans*-3-phenyloxiran-2-methanol and (2*S*,3*S*)-*trans*-3-phenyloxiran-2-methanol, respectively. (*R*)-Phenylglycol and (*S*)-1-phenylethanol have previously been reported to be substrates also for human liver SDH [24]. A role for SDH in xenometabolism is also suggested by the finding that 3-butene-1,2-diol, a metabolite of the carcinogenic petrochemical 1,3-butadiene and a substrate for liver ADH [55], is also a substrate for SDH. While racemic 3-butene-2-ol is as expected not a substrate (2*R*)-3-butene-1,2-diol, but not the (2*S*)-isomer, has an activity with SDH ($k_{\text{cat}} = 2.8 \text{ s}^{-1}$, $K_m = 14 \text{ mM}$) similar to that of (2*R*)-1,2-butanediol (Table 2). The concept that compounds other than polyols are substrates for SDH *in vivo* is supported by the finding that an unidentified non-polyol component contributes to the apparent differences between sorbitol levels determined enzymatically and chromatographically [56].

Like the related mammalian ADH, SDH is a *pro-R* specific dehydrogenase [4]. Benner and co-workers have correlated K_{eq} of a dehydrogenase-catalysed reaction with the stereospecificity of hydride transfer, separating enzymes with $\text{p}K_{\text{eq}} > 11$, grouped as *pro-R* specific, from those that are *pro-S* specific with $\text{p}K_{\text{eq}} < 11$ [57]. Maret has pointed out that sorbitol/D-fructose, with $\text{p}K_{\text{eq}} = 8.4$, may not be the physiologically important substrate

pair of SDH [24]. However, also for the interconversion of most other substrates $\text{p}K_{\text{eq}} < 11$, and if the aforementioned hypothesis prevails this suggests that SDH may have evolved under little selective pressure favouring one stereochemical outcome over the other, as seems to be the case for ADH [57].

Recently, the potent SDH inhibitor 2-hydroxymethyl-4-(4-*N,N*-dimethylaminosulphonyl-1-piperazino)pyrimidine (SDI 158) was shown to have clinically beneficial effects in diabetic rat tissues [10,14]. At present, many investigators are attempting the synthesis of potent and specific SDH inhibitors for prospective clinical trials. The substrate specificity of this enzyme is a premise for rational drug design, because substrate analogues can be expected to be optimally potent and specific inhibitors. It has been suggested that SDI 158 binds in the enzyme active site as a substrate analogue [10]. Derivatives of substrates, including polyols, deoxy polyols and aromatic alcohols, with an inhibitory group such as thiolate [14] in the reactive 2-position (e.g. 2-thiosorbitol), can be expected to be exceedingly potent and specific enzyme inhibitors. Due to metabolic stability and low toxicity, polyol based inhibitors should be particularly interesting.

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