Reversible inhibition of sheep liver sorbitol dehydrogenase by the antidiabetogenic drug 2-hydroxymethyl-4-(4-*N*,*N*-dimethylaminosulfonyl-1-piperazino) pyrimidine

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Abstract The mechanism of the inhibition of sheep liver sorbitol dehydrogenase by the novel antidiabetogenic drug 2-hydroxymethyl-4-(4-*N*,*N*-dimethylaminosulfonyl-1-piperazino)pyrimidine has been investigated by steady-state kinetics over the range pH 5–10. The pyrimidine derivative exhibits mixed inhibition with respect to sorbitol, fructose and coenzyme, due to the formation of enzyme-inhibitor and enzyme-NAD(H)-inhibitor complexes. The formation of each of the binary and ternary complexes is inhibited by protonation and deprotonation of groups which, in the enzyme-inhibitor complex, have p*K* values of 6.6 and 8.0, respectively.

Key words: Sorbitol; Diabetes; SDI 158; Zinc; Sheep liver

1. Introduction

Sorbitol dehydrogenase (SDH) is a homotetrameric zinc enzyme of 38 kDa subunits present in most mammalian tissues [1]. The enzyme belongs to a family of dehydrogenases/reductases which also includes several classes of alcohol dehydrogenases (ADH), ζ-crystallin and numerous other proteins [2]. Although data on the three-dimensional structure and functionally significant residues, apart from the zinc ligands Cys^{43}, His^{68} and Glu^{149} [3], are unavailable, a model has been constructed for the subunit structure of sheep liver SDH on the basis of the crystal structure of the related horse liver ADH and sequence alignment [4]. Crystallographic studies on sheep liver SDH are in progress (H. Eklund, unpublished results).

Aldose reductase (AR) and SDH constitute the sorbitol, or polyol, pathway [5], which catalyzes the conversion of glucose to fructose via sorbitol:

$$\text{glucose} + \text{NADPH} + \text{H}^+ \overset{\text{AR}}{\rightleftharpoons} \text{sorbitol} + \text{NADP}^+$$

$$\text{sorbitol} + \text{NAD}^+ \overset{\text{SDH}}{\rightleftharpoons} \text{fructose} + \text{NADH} + \text{H}^+.$$

The increased flux via the pathway in hyperglycemia, as in poorly controlled diabetes mellitus, has been implicated in the etiology of diabetic complications [6]. Pathogenesis in some tissues, such as lens, has been attributed to sorbitol accumulation and concomitant osmotic anomalies [7], and AR inhibitors have been used for many years in the treatment of diabetic complications [8]. However, recently the clinically beneficial effects of the novel SDH inhibitor 2-hydroxymethyl-4-(4-*N*,*N*-dimethylaminosulfonyl-1-piperazino) pyrimidine (SDI 158) in diabetic rat tissues were reported [9,10]. Although the inhibition of SDH aggravated sorbitol accumulation it also alleviated, or attenuated, various complications, including impaired nerve conduction and vascular dysfunction in nerve, aorta and ocular tissues, without side effects. This implies that pathogenesis in these tissues is more closely associated with oxidation of sorbitol to fructose, catalyzed by SDH, than with reduction of glucose to sorbitol. Thus, an elevated NADH/NAD ratio appears to be the single most important determinant of pathogenesis in various diabetic tissues [10]. SDI 158 has recently also been reported to inhibit the SDH-catalyzed oxidation of sorbitol to fructose, thereby inhibiting glucokinase, in non-diabetic rat hepatocytes [11]. Whereas SDI 158 weakly inhibits AR, the compound has no effect on the activities of numerous other enzymes, including hexokinase, glutathione reductase, glucose-6-phosphate dehydrogenase, lactate dehydrogenase and alcohol dehydrogenase [9,10], indicating that SDI 158 is a specific inhibitor of SDH. The related compound 2-methyl-4-(4-*N*,*N*-dimethylaminosulfonyl-1-piperazino) pyrimidine (SDI 157) inhibits SDH poorly in vitro, and appears to be a prodrug [10].

At present, many investigators are attempting the synthesis of potent and specific SDH inhibitors for prospective use as drugs. SDI 158 is the only inhibitor clinically evaluated thus far and, in view of the few and inconclusive kinetic data previously published [9,10], the object of the present study was to investigate the mechanism for the inhibition of SDH by this compound.

2. Materials and methods

2.1. Materials

Lyophilized sheep liver SDH was obtained from Boehringer and stock enzyme solutions were prepared by equilibrium dialysis, as described previously [12]. The dialysed enzyme solutions were homogeneous upon starch gel electrophoresis and had a purity ≥98%. The concentration of active enzyme in solution was determined spectrophotometrically, as previously described [12]. Stock enzyme solutions were stable for several days at 4°C.

Sorbitol, D-fructose, NAD, NADH and piperazine were obtained from Sigma. All reagents were of p.a. quality and solutions were prepared in Milli-Q water. NADH was dissolved in 10 mM sodium phosphate, pH 7.4, because of the instability of the coenzyme in acid media. SDI 158 (p*K* = 5.9) was supplied by Hoechst AG, Frankfurt am Main. The compound shows high solubility in water and is stable in aqueous solution. Stock solutions of SDI 158 were kept in the dark at 4°C.

2.2. Kinetic measurements

The initial rate of NAD-linked oxidation of sorbitol or NADH-linked reduction of fructose was determined at 23.5°C by measuring the change in absorbance at 340 nm due to NAD reduction or NADH oxidation, using a Philips PU 8675 spectrophotometer connected to

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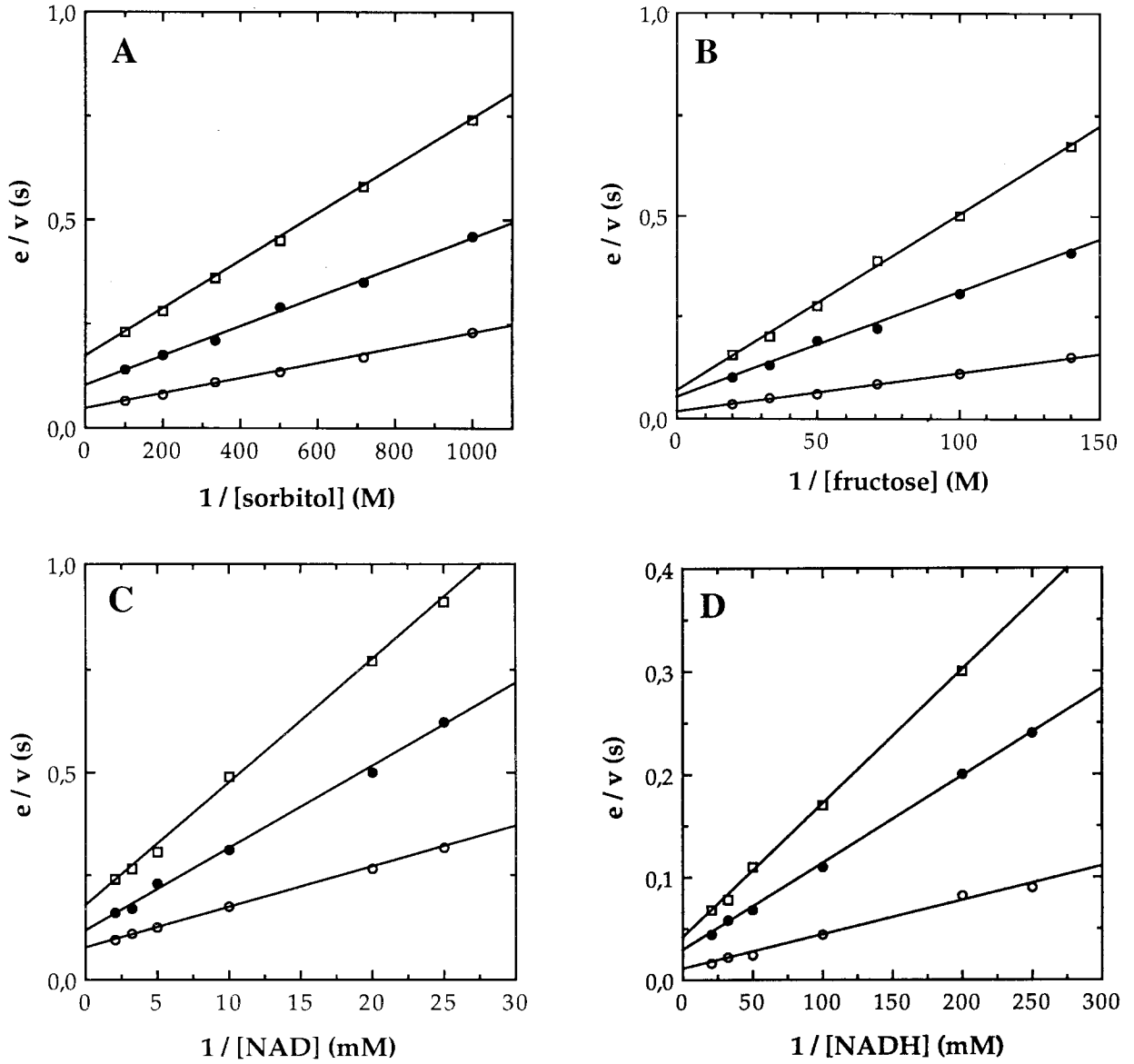


Fig. 1. Reversible inhibition of sheep liver sorbitol dehydrogenase by SDI 158 at pH 7.4. A: Double-reciprocal plots showing the effect of SDI 158 on the initial rate of sorbitol oxidation. [NAD] = 500 μM; without inhibitor (○), [SDI 158] = 0.50 μM (●), 1.0 μM (□). B: Double-reciprocal plots showing the effect of SDI 158 on the initial rate of fructose reduction. [NADH] = 50 μM; without inhibitor (○), [SDI 158] = 1.0 μM (●), 2.0 μM (□). C: Double-reciprocal plots showing the effect of SDI 158 on the initial rate of sorbitol oxidation. [sorbitol] = 10 mM; without inhibitor (○), [SDI 158] = 0.60 μM (●), 1.2 μM (□). D: Double-reciprocal plots showing the effect of SDI 158 on the initial rate of fructose reduction. [fructose] = 100 mM; without inhibitor (○), [SDI 158] = 1.0 μM (●), 2.0 μM (□). In each case, e denotes the concentration of enzyme (M) and v is the initial velocity (m s^{-1}).

an ABB Goertz SE 120 recorder. Reversible kinetics were performed by varying the concentration of sorbitol, fructose, NAD or NADH, with a constant concentration of the other reaction partner, in a total volume of 3.0 ml of 50 mM sodium phosphate, pH ≤ 8.0, or 50 mM glycine/NaOH, pH ≥ 9.0 [13]. Two different [SDI 158] were used in each case in order to determine the kinetic inhibition constants. The relative standard deviations for the kinetic constants obtained by regression analysis of the primary data were ≤10%.

3. Results and discussion

SDI 158 is a potent mixed inhibitor of the SDH-catalyzed oxidation of sorbitol and reduction of fructose. Fig. 1 shows the mixed inhibition patterns in double-reciprocal plots at pH 7.4 with varied concentrations of sorbitol (a), fructose (b), NAD (c) and NADH (d). In each case, the inhibition constants derived from the slope and intercept effects (see below) with two different inhibitor concentrations agreed to within the experimental error, indicating a simple, linear relationship between [SDI 158] and the inhibitory effect.

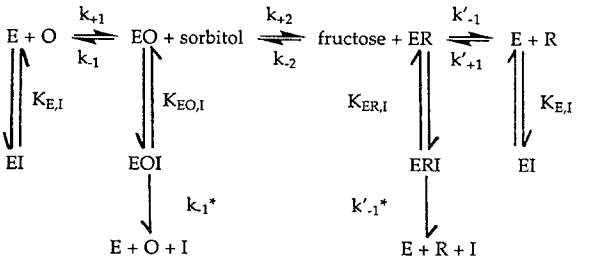
Sorbitol oxidation and fructose reduction catalyzed by sheep liver SDH follow a compulsory ordered (Theorell-Chance) kinetic mechanism, with only the coenzymes forming binary complexes with the free enzyme in each direction and the enzyme-coenzyme product dissociation as the rate-limiting step for the overall reaction [14]. The kinetic mechanism of sheep liver SDH can be depicted as in scheme 1:

$$E + O \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} EO + \text{sorbitol} \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} \text{fructose} + ER \underset{k'_{+1}}{\overset{k'_{-1}}{\rightleftharpoons}} E + R$$

where E = enzyme, O = NAD and R = NADH. The initial rate equation for the mechanism may be written: $e/v = \Phi_0 + \Phi_1/[NAD] + \Phi_2/[\text{sorbitol}] + \Phi_{12}/[NAD][\text{sorbitol}]$, where the kinetic coefficients (Φ), which previously have been determined over the range pH 5–10 [14], are related to the rate constants (k) in scheme 1 through $\Phi_0 = 1/k'_{-1}$, $\Phi_1 = 1/k_{+1}$, $\Phi_2 = 1/k_{+2}$ and $\Phi_{12} = k_{-1}/k_{+1}\ k_{+2}$. Because of the symmetry of the mechanism, the initial rate equations for the forward and reverse reactions are interchangeable by the insertion or deletion of primes on the Φ coefficients.

The slope effects vs. varied [NAD] reflect formation of an enzyme-inhibitor (EI) binary complex (Fig. 1C). Because the Φ_1 and Φ_{12} terms are similarly affected by the binding of inhibitor to the free enzyme, it follows that $K_{E,I} = [I]/((S_i/S_0)-1)$, where S_i and S_0 are the slopes in double-reciprocal plots with and without inhibitor, respectively. The slope effects vs. varied [sorbitol] reflect formation of an EI and/or enzyme-NAD-inhibitor (EOI) complex (Fig. 1A), affecting the Φ_{12} and Φ_2 terms, respectively. When $K_{E,I}$ is known, $K_{EO,I}$ can be computed [15] using the relationship $K_{EO,I} = [I]/[(1/\Phi_2)\ (S_i-(\Phi_{12}/[NAD])(1+[I]/K_{E,I}))-1]$. The intercept effects vs. varied [NAD] reflect formation of an EOI and/or enzyme-NADH-inhibitor (ERI) complex affecting the Φ_2 and Φ_0 terms, respectively. With sorbitol as the varied substrate, the intercept effects represent an EI and/or ERI complex affecting, respectively, the Φ_1 and Φ_0 terms. When $K_{E,I}$ and $K_{EO,I}$ are known, $K_{ER,I}$ can be computed [15] using the relationships $K_{ER,I} = [I]/[(1/\Phi_0)\ (I_i-(\Phi_2/[\text{sorbitol}])(1+[I]/K_{EO,I}))-1]$ for fixed [sorbitol] and $K_{ER,I} = [I]/[(1/\Phi_0)\ (I_i-(\Phi_1/[NAD])(1+[I]/K_{E,I}))-1]$ for fixed [NAD], where I_i is the inhibited intercept in a double-reciprocal plot. Due to the symmetry of the mechanism (scheme 1), the inhibited slopes and intercepts with varied [NADH] represent $K_{E,I}$ and $K_{EO,I}/K_{ER,I}$, respectively. With fructose as the varied substrate, the inhibited slopes represent $K_{E,I}/K_{ER,I}$ and the inhibited intercepts are $K_{E,I}/K_{EO,I}$. Whereas $K_{E,I}$ measures the affinity of SDI 158 for the free enzyme, $K_{EO,I}$ and $K_{ER,I}$ measure the affinity of the inhibitor for the enzymic binding site in the EO and ER complexes, respectively. Likewise, $K_{EI,O}$ and $K_{EI,R}$ measure the affinity of the coenzymes for the EI complex. The latter constants were calculated using the relationships $K_{EI,O} = K_{EO,I} \cdot K_{E,O}/K_{E,I}$ and $K_{EI,R} = K_{ER,I} \cdot K_{E,R}/K_{E,I}$. The values of $K_{E,O}$ and $K_{E,R}$ have previously been determined over the range pH 5–10 [14].

Table 1 lists the kinetic constants for the mixed inhibition of sheep liver SDH by DI 158 over the range pH 5.5–9.9. $K_{E,I}$ always represents the inhibited slopes with respect to NAD(H), and as the EOI complex is not kinetically significant at pH < 7.4, the inhibited slopes with respect to sorbitol also represent $K_{E,I}$ at low pH. The similar constants derived from the intercept effects with respect to NAD and sorbitol represent $K_{ER,I}$ in both cases. The proposed kinetic mechanism for the mixed inhibition of sorbitol oxidation and fructose reduction by SDI 158 can be depicted as follows:



In each direction, SDI 158 inhibits the steady-state rate of catalysis by combining with the free enzyme and with the holoenzyme substrate, and the additional binding of inhibitor to the holoenzyme product gives a ternary complex dissociating more slowly than the normally rate-limiting enzyme-coenzyme product dissociation (i.e. $k'_{-1}* < k'_{-1}$ or $k_{-1}* < k_{-1}$).

Previously, SDI 158 was reported to inhibit sorbitol oxidation uncompetitively with respect to sorbitol and NAD with, in both cases, an inhibition constant of 0.19 μM [10], which should be $K_{ER,I}$. Although no details regarding pH or experimental procedures were given, this appears to agree with the present results, insofar as the same value for the inhibition constant ($K_{ER,I}$) was obtained from the intercept effects with respect to both NAD and sorbitol. The present study also accords with the inhibition patterns of Geisen and co-workers [9] who, on the basis of Dixon plots, reported an apparent inhibition constant of 3.7 μM at pH 9.5 with sorbitol as the varied substrate. Double-reciprocal plots of the latter data reveal mixed inhibition with respect to sorbitol, indicating formation of EI, EOI and ERI complexes. However, their previously reported competitive inhibition patterns (K_i = 92 μM) against varied [fructose] in triethanolamine buffer, pH 7.4, do not accord with the present study.

Table 1
Reversible inhibition of sheep liver sorbitol dehydrogenase by SDI 158

pH	$K_{E,I}$ (μM)	$K_{EO,I}$ (μM)	$K_{ER,I}$ (μM)	$K_{EI,O}$ (μM)	$K_{EI,R}$ (μM)	$K_{E,I}/K_{EO,I}$	$K_{E,I}/K_{ER,I}$
5.5	5.2	n.s.	2.3	n.s.	0.71	< 1	2.3
6.1	2.0	n.s.	0.88	n.s.	1.2	< 1	2.3
6.8	1.1	n.s.	0.71	n.s.	3.2	< 1	1.5
7.4	0.65	0.29	0.58	542	6.4	2.2	1.1
8.0	1.1	0.23	0.55	220	4.5	4.8	2.0
9.0	2.0	0.46	0.61	209	3.4	4.3	3.3
9.9	2.0	1.4	0.80	343	5.4	1.4	2.5

$K_{E,I}$, $K_{EO,I}$ and $K_{ER,I}$ measure the affinity of SDI 158 for the free enzyme, the enzyme-NAD complex and the enzyme-NADH complex, respectively, and were derived from the slope and intercept effects in double-reciprocal plots with varied concentrations of NAD(H), sorbitol or fructose (see main text). The constants were determined in 50 mM sodium phosphate, pH ≤ 8.0, or 50 mM glycine/NaOH, pH ≥ 9.0. $K_{EI,O}$ and $K_{EI,R}$ measure the affinity of the coenzymes for the enzyme-inhibitor complex, and were calculated using the relationships $K_{EI,O} = K_{EO,I} \cdot K_{E,O}/K_{E,I}$ and $K_{EI,R} = K_{ER,I} \cdot K_{E,R}/K_{E,I}$. The ratios $K_{E,I}/K_{EO,I}$ (= $K_{E,O}/K_{EI,O}$) and $K_{E,I}/K_{ER,I}$ (= $K_{E,R}/K_{EI,R}$) reflect the relative stability of the ternary and binary complexes with SDI 158. n.s. = not kinetically significant.

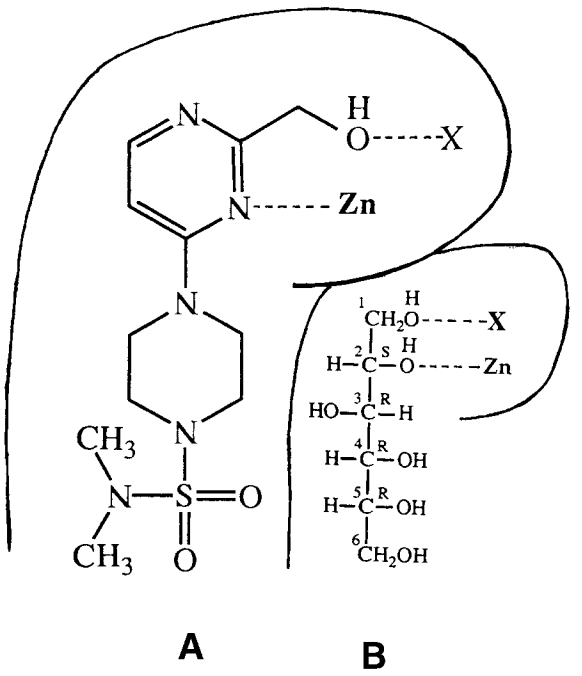


Fig. 2. Schematic representation of putative interactions of SDI 158 with sheep liver sorbitol dehydrogenase. Zn denotes the catalytic zinc atom, liganded by Cys^{43}, His^{68} and Glu^{149} [3], and X represents an enzymic group suggested to be Glu^{154} and/or Lys^{293} (see text). In the enzyme bound binary or ternary complex, SDI 158 is coordinated to zinc via N3 of the pyrimidine ring (A). Thus bound, SDI 158 mimics sorbitol (B), which binds with the C2-OH coordinated to zinc and the C1-OH interacting with Glu154 and/or Lys293 [4].

Kinetic data and the enzyme structure model [4] enable a rationalization of the interactions of SDI 158 with enzymic groups. As in the case of CNAD, a potent inhibitor of horse liver ADH [16], the primary interaction of inhibitory nitrogen, oxygen and sulphur nucleophiles with the related SDH can be expected to involve the catalytic zinc atom. The finding that piperazine (10 mM), dimethylsulfoxide [13] and various sulfonamides [12] have no significant inhibitory effect on SDH activity implies that the pyrimidine moiety of SDI 158 is coordinated to zinc in the binary and ternary complexes. However, various pyrimidine derivatives and other heterocyclics are comparatively poor inhibitors of SDH [12,13] and, hence, the potent inhibition by SDI 158 indicates that factors other than binding to the catalytic metal are also involved in complex formation with the enzyme. Two aspects of the structure of SDI 158 appear important in this respect. As shown by the poor inhibition by 2-methyl-4-(4-*N*,*N*-dimethylaminosulfonyl-1-piperazino) pyrimidine in vitro [10], the 2-hydroxymethyl substituent of SDI 158 is clearly a determinant for pyrimidine binding to zinc. According to the SDH structure model, Glu^{154} and/or Lys^{293} forms a specific hydrogen bond with the primary hydroxyl group adjacent to the C2-OH of sorbitol, enabling the latter group to be poised for reaction at the catalytic zinc in the enzyme-NAD-sorbitol complex [4]. This is supported by site-directed mutagenesis studies [17], and by the fact that *Bombyx mori* SDH, which has a Gln residue in this position, shows an extremely low affinity for sorbitol [18]. A similar interaction appears involved also in the binding of various reversible inhibitors to the enzyme [12,13]. Hence, the binary and ternary complexes of sheep liver SDH with SDI 158 can be considered to involve an interaction of the 2-hydroxymethyl moiety of the inhibitor with Glu^{154} and/or Lys^{293}, and monodentate binding of the N3 of the pyrimidine moiety to zinc (Fig. 2). Thus bound at the bottom of the catalytic site, SDI 158 mimics a substrate. Furthermore, hydrophobic effects are likely to be involved in the binding of the largely non-polar ligand, as appears to be the case also with various substrates [12], reversible inhibitors [12,13] and affinity labelling reagents [19].

The pH dependence of the mixed inhibition of SDH by SDI 158 reflects protonation/deprotonation of the inhibitor ($pK = 5.9$) and of enzymic groups interacting with the inhibitor in the bound complexes. Formation of the EI, EOI and ERI complexes is inhibited by protonation and deprotonation of groups with, in the EI complex, pK values of 6.6 (± 0.2) and 8.0 (± 0.2), respectively. The former group is considered to be the pyrimidine moiety of SDI 158, perturbed by 0.7 unit upon formation of the EI complex. Protonation of this group removes its metal chelating capability and, hence, inhibits complex formation with the enzyme at low pH. This parallels the pH dependence of the metal-directed affinity labelling of SDH by 2-bromo-3-(5-imidazolyl) propionic acid, which has been attributed to protonation of the imidazole moiety of the reagent ($pK = 7.0$) inhibiting its binding to zinc at low pH [20]. The pK 8.0 group is considered to be the water ligand to the catalytic zinc as, at high pH, a zinc bound hydroxide ion can be expected to repel the nitrogen nucleophile and thus inhibit binding. It appears that the pK of the enzymic group involved is perturbed to above 10 in the ERI complex. In the case of liver ADH, where the pK of the zinc-water ionization is perturbed to 11.2 upon binding of NADH [21], the differential effects of NAD and NADH on the ionization properties of the zinc-water ligand reflects stabilization and destabilization of zinc bound hydroxide by NAD and NADH, respectively. The previously reported mixed inhibition of SDH by thionucleosides shows a similar pH dependence [12], suggesting a similar mechanism involving purine or pyrimidine binding to zinc in the binary or ternary complexes.

Table 1 lists the ratios $K_{E,I}/K_{EO,I}$ ($= K_{E,O}/K_{EI,O}$) and $K_{E,I}/K_{ER,I}$ ($= K_{E,R}/K_{EI,R}$) over the range pH 5.5–9.9. $K_{ER,I} < K_{E,I}$ and $K_{EI,R} < K_{E,R}$, reflecting stabilization of the ternary in relation to the binary complexes. Similarly, the ternary complexes with NAD are stabilized at pH ≥ 7.4. This stabilization is likely to stem from a coenzyme- induced enzyme conformation change facilitating inhibitor binding in the catalytic site, as well as steric effects [12]. However, the tight binding of SDI 158 to the free enzyme suggests that a coenzyme-induced conformation change may not be a prerequisite for binding. Interestingly, this structural transition upon coenzyme binding to the enzyme is induced by the nicotinamide part of NAD(H) [22]. The structurally similar pyrimidine moiety of SDI 158 may enable tight binding in the catalytic domain of SDH, without prior coenzyme binding, by inducing a similar structural transition. The destabilization of the EOI complex at pH < 7.4 is likely to stem from unfavorable electrostatic interaction of the bound coenzyme with the positively charged pyrimidine moiety of the inhibitor at low pH. Conversely, the stabilization of the EOI complexes with various thiol inhibitors has been attributed to thiolate inhibiting the release of coenzyme, and vice versa, from the ternary complexes [12].

The mixed inhibition of SDH by 6-thioinosine and 6-thioguanosine was previously interpreted in terms of EOI and

ERI ternary complexes giving rise to slope and intercept effects, respectively, in double-reciprocal plots with varied concentrations of sorbitol [12]. It is likely, that the inhibition by the thionucleosides also involves formation of EI complexes and, hence, parallels that seen with SDI 158. Various other purine and pyrimidine derivatives, as well as polyol substrate analogues (e.g. 2-thiosorbitol), are expected to be potent inhibitors of SDH and, hence, interesting for further study and prospective clinical trials. As in the case of aldose reductase [23], the elucidation of the sheep liver SDH crystal structure will enable design of enzyme inhibitors of optimal potency and specificity.

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