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Inhibition of sorbitol dehydrogenase by nucleosides and nucleotides

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ABSTRACT

Sorbitol dehydrogenase inhibitors have been found to prevent, or alleviate, various secondary complications of diabetes mellitus. In the present study, the effects of nucleosides and nucleotides on the rate of sorbitol oxidation catalyzed by the sheep liver enzyme were studied by steady-state kinetics at pH 7.4. Various such compounds, including ATP and the 2′-deoxy-analogues of ATP, ADP and AMP, reversibly inhibit enzyme activity by formation of enzyme–coenzyme-inhibitor ternary complexes. In each case, no deviations from linearity were seen in the double-reciprocal plots using sorbitol or NAD^+ as the varied substrate and there was a linear relationship between inhibitor concentration and the observed inhibitory effects. Sorbitol was docked into a model of the sheep SDH-NAD^+ complex based upon the structure of the human SDH-NAD^+ holoenzyme. The resulting structure of the ternary complex of sheep SDH, NAD^+ and sorbitol (PMDB ID code PM 0078068) shows that the reactive C-2 hydroxyl group of sorbitol is oriented toward the 4′-position of the nicotinamide moiety of the coenzyme, and that the adjacent primary hydroxyl group of sorbitol interacts with the catalytic zinc. The results indicate that the ribose moiety of the inhibitor structures is an important determinant for the observed effects. Specifically, the 2′-position of the ribose ring exerts an effect with respect to inhibitor potency.

1. Introduction

Sorbitol dehydrogenase (SDH) belongs to a protein superfamily that also includes several classes of alcohol dehydrogenases (ADH) [1,2], and is widely distributed in mammalian tissues [1,3,4]. The functional mammalian SDH molecule is a homotetrameric zinc enzyme of 38-kDa subunits, and at present the crystal structures of rat, sheep and human SDH have been characterized [5–7]. Each enzyme subunit contains one active site in close proximity to the tetramer interface, and the catalytic zinc atom of each subunit is coordinated by Cys43, His68, Glu69 (sheep enzyme) and a water molecule [5–7].

SDH catalyzes nucleophilic attack by the C-2 hydrogen of sorbitol on the *re*-side of C-4 of the nicotinamide moiety of NAD^+, giving D-fructose and NADH as products [1]. The overall reaction is compulsory ordered, with the initial binding of coenzyme to the free enzyme being a prerequisite for ternary complex formation and catalysis [8,9]. The sheep liver enzyme has previously been characterized in terms of the kinetic mechanism [8,9], substrate specificity [10,11], affinity labeling [12,13] and reversible inhibition [14,15].

The sorbitol, or polyol, pathway constitutes a bypass to glycolysis by converting glucose to D-fructose via sorbitol by the combined actions of aldose reductase (AR) and SDH [1]:

$$\text{Glucose} + \text{NADPH} + \text{H}^+ \rightarrow^{\text{AR}} \text{sorbitol} + \text{NADP}^+$$

$$\text{Sorbitol} + \text{NAD}^+ \rightarrow^{\text{SDH}} \text{D-fructose} + \text{NADH} + \text{H}^+$$

It appears that normally the operation of the pathway plays only a minor physiological role [1]. However, increased metabolic flux via AR and SDH under hyperglycemic conditions has been implicated in the etiology of various secondary complications of diabetes, such as cataractogenesis, neuropathy, nephropathy and cardiomyopathy [16,17]. The molecular basis of such complications generally includes depletion of cellular NADPH, and a perturbation of the cellular [NAD^+]/[NADH] ratio. The increased level of NADH in the diabetic state may, apart from effects on many metabolic pathways, also lead to oxidative stress (“pseudohypoxia”). The biochemical changes involved include depletion of cellular reduced glutathione and accumulation of harmful reactive oxygen species and other cytotoxic compounds, compromising macromolecular structure and function as well as membrane integrity [18–20].

Abbreviations: SDH, sorbitol dehydrogenase; ADH, alcohol dehydrogenase; AR, aldose reductase; E, enzyme; I, inhibitor; EI, enzyme-inhibitor; EO, enzyme-NAD^+; ER, enzyme-NADH; EOI, enzyme-NAD^+-inhibitor; ERI, enzyme-NADH-inhibitor; SDI, sorbitol dehydrogenase inhibitor.

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It has been known for many years that AR inhibitors may have clinically beneficial effects in diabetics [21]. Geisen and coworkers demonstrated that the potent and specific SDH inhibitor 2-hydroxymethyl-4-(4-N,N-dimethylaminosulfonyl-1-piperazino) pyrimidine (SDI 158) may alleviate neural dysfunction in diabetic rats [22]. At present numerous other compounds, including piperazino-pyrimidines, pyrimidine–triazines and triazine–triazines, have been synthesized and tested with respect to their SDH inhibitory effects *in vitro* as well as *in vivo* [17,23–26]. Many of these SDH inhibitors show significantly improved pharmacological properties compared with the original compound SDI 158 [17,22–26].

Several dehydrogenases are known to be inhibited by ATP, ADP and AMP, i.e. glyceraldehyde-3-phosphate dehydrogenase, alcohol dehydrogenase and lactate dehydrogenase [27]. Based on a relatively weak literature lead [14], the purpose of this study was to specifically screen nucleosides and nucleoside phosphates with respect to their eventual effects on SDH activity.

2. Materials and methods

2.1. Materials

Lyophilized sheep liver SDH, sorbitol, NAD^+ and the various compounds screened were obtained from Sigma–Aldrich. All reagents used were of *pro analysi* quality and solutions were prepared in milli-Q water. Glassware was routinely rinsed using 6 M nitric acid. Enzyme stock solutions were prepared by dialysis against 0.1 M sodium phosphate, pH 7.4 [8]. The concentration of active enzyme in solution was determined as described previously [8].

2.2. Enzyme kinetics

The initial rate of the NAD^+ linked oxidation of sorbitol was determined spectrophotometrically at 23.5 °C, by measuring the increase in absorbance at 340 nm (ΔA_{340} s^{-1}) due to NADH formation using a Hitachi U-2000 spectrophotometer with a built-in recorder. Kinetic measurements were made by varying the concentration of sorbitol or NAD^+ at a constant saturating concentration of the other reaction partner in a total volume of 1.0 ml of 50 mM sodium phosphate, pH 7.4, or 50 mM glycine/NaOH, pH 9.9. The kinetic data were analyzed by nonlinear regression using the *SigmaPlot* program, and the kinetic inhibition constants were determined with SD $\leqslant$ 10%.

2.3. Enzyme docking

The structure of sheep liver SDH (pdb code 3QE3) was superimposed on the structure of human SDH in complex with NAD^+ (pdb code 1PL8). Human and sheep SDH were superimposed with a RMSD of 0.63 Å for main chain atoms. The coordinates of the human enzyme were removed in order to create a model of sheep SDH with NAD^+. This model was prepared for docking with the Protein Preparation wizard, part of the Schrödinger program package, using default parameters. The zinc atom was assigned a formal charge of +1 to mimic charge delocalization, and the coordinating amino acid residues were assigned standard charges. This procedure has previously been found to be advantageous for docking to zinc enzymes [28–30]. A grid was centered on the metal atom and used for scoring sorbitol orientations in the active site. Glide [31] was used to dock sorbitol into the active site of sheep SDH in the presence of NAD^+, following the extra precision (XP) protocol in Glide [32].

3. Results

3.1. Enzyme inhibition

Table 1 lists the kinetic inhibition patterns and inhibition constants obtained for the various nucleosides and nucleoside phosphates used in the present study with sorbitol as the varied substrate. It was found, that the inhibitory effects were significantly more pronounced at pH 7.4 than at the pH 9.9 optimum [9] for sorbitol oxidation. In Table 1, it is noticeable that all compounds tested, apart from ATP, show either uncompetitive or mixed inhibition with respect to sorbitol.

None of the compounds activated sorbitol oxidation, and no deviations from linearity were seen in any of the double-reciprocal plots. Also, there was in each case a simple linear relationship between the inhibitor concentration used and the observed inhibitory effects. This is exemplified by the uncompetitive inhibition by Guanosine-2,3-di-P (Fig. 1). In this case, a K_{ERI} value of 1.0 mM was derived from the intercept effects irrespective of the inhibitor concentration used (1–10 mM).

Fig. 2(A–D) shows double-reciprocal plots of the inhibition of SDH by 2-deoxy-adenosine-5-P or Guanosine-2-P with varied [sorbitol] or [NAD^+]. Whereas the former compound inhibits sorbitol oxidation in an uncompetitive manner (Fig. 2A), the latter exhibits mixed inhibition (Fig. 2C) of enzyme activity upon varying the [sorbitol]. However, in both cases the inhibition patterns are uncompetitive with respect to NAD^+ (Fig. 2B and D). The intercept or

Table 1
Reversible inhibition of sheep liver sorbitol dehydrogenase by nucleosides and nucleotides at pH 7.4. Measurements were made at 23.5 °C by varying the [sorbitol] at a fixed [NAD^+] of 500 μM in 1.0 ml of 50 mM phosphate buffer, pH 7.4. Each compound was tested at concentrations within the 1–10 mM range. The dissociation constant of the enzyme-NAD^+-inhibitor complex (K_{EOI}) was derived from the slope effects seen in the double-reciprocal plots with sorbitol as the varied substrate using the relationship $S_i/S_0 = (1+[I]/K_{EOI})$ where S_i and S_0 denote the slope with or without inhibitor (I) present, respectively. Likewise, the dissociation constant of the enzyme-NADH-inhibitor complex (K_{ERI}) was derived from the intercept effects seen in the double-reciprocal plots with sorbitol as the varied substrate using the relationship $I_i/I_0 = (1+[I]/K_{ERI})$ where, respectively, I_i and I_0 denote the intercept with or without inhibitor present [14,15,33]. C, UC and M denote competitive, uncompetitive and mixed inhibition, respectively, of sorbitol oxidation. NSI; no significant inhibition.

		K_{EOI} mM	K_{ERI} mM
Adenosine-5-tri-P	C	9.2	
2-Deoxy-adenosine-5-tri-P	UC		6.6
Adenosine-5-di-P	NSI		
2-Deoxy-adenosine-5-di-P	UC		6.5
Adenosine-5-mono-P	NSI		
2-Deoxy-adenosine-5-mono-P	UC		1.3
Guanosine-5-tri-P	NSI		
Guanosine-5-mono-P	NSI		
2-Deoxy-guanosine-5-mono-P	UC		2.0
Guanosine-3-mono-P	NSI		
Guanosine-2-mono-P	MI	1.1	0.30
Guanosine-2,3-di-P	UC		1.0
2-Deoxy-guanosine	MI	2.4	2.0
Inosine-5-mono-P	MI	1.8	1.2
2-Deoxy-inosine-5-mono-P	UC		4.5
2-Deoxy-inosine	NSI		
2-Deoxy-cytidine-5-mono-P	UC		1.7
2-Deoxy-cytidine-3-mono-P	NSI		
Thymidine-5-mono-P	NSI		
Thymidine-3-mono-P	NSI		
Thymidine	UC		8.4
Uridine-5-tri-P	NSI		
Uridine-5-mono-P	NSI		
2-Deoxy-uridine-5-mono-P	UC		2.3
Uridine-3-mono-P	NSI		
Uridine-2-mono-P	UC		0.80
2-Deoxy-uridine	NSI		

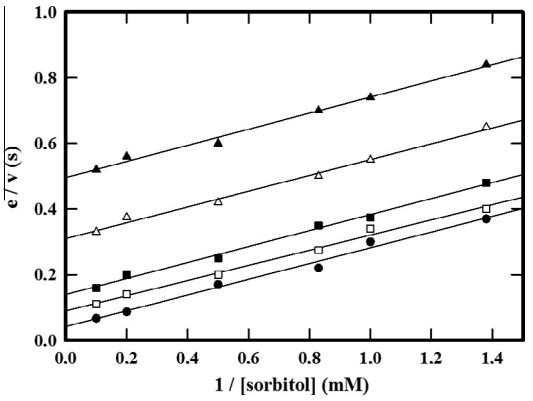

Fig. 1. Reversible inhibition of sheep liver sorbitol dehydrogenase by Guanosine-2,3-di-P at pH 7.4. Double-reciprocal plots showing the effect of Guanosine-2,3-di-P on the initial rate of sorbitol oxidation at a constant saturating [NAD^+] = 500 μM. Without inhibitor (●); [inhibitor] = 1.0 mM (□), 2.0 mM (■), 6.0 mM (△), 10 mM (▲). In each case e denotes the molar concentration of active enzyme, and v is the initial rate of the reaction in terms of Δ [NADH] per second ($M\ s^{-1}$).

slope effects are interpreted in terms of the formation of enzyme-NAD^+-inhibitor (EOI) and/or enzyme-NADH-inhibitor (ERI) ternary complexes. Thus, the inhibition constants listed in Table 1 are denoted K_{EOI} or K_{ERI}.

The inhibitors tested show higher affinity for the enzyme-NADH than for the enzyme-NAD^+ complex. The 2′-deoxy arrangement of ribose is evidently favorable with respect to inhibitor potency. Furthermore, the presence of a phosphate group in the 2′-position of ribose is positively correlated with increasing inhibitory effect. Indeed, the two most potent inhibitors are Guanosine-2-P and Uridine-2-P.

3.2. Enzyme docking

Fig. 3 depicts NAD^+ and sorbitol docked into the active site of the sheep SDH molecule. Sorbitol was found to make favorable interactions directly with the active site zinc atom with an interatomic distance of 2.1 Å between the C-1 oxygen atom of sorbitol and zinc. The reactive C-2 oxygen atom of sorbitol is directed toward the 4′-position of the nicotinamide moiety of NAD^+. Water molecules were removed prior to docking sorbitol into the enzyme-NAD^+ complex. However, subsequent to docking the inclusion of zinc-water or zinc-hydroxide placed this group at a distance of 1.9 Å from the C-1 oxygen of sorbitol. Fig. 4(A and B) shows ATP docked into the coenzyme binding domain of sheep SDH. The structure was manually generated by removing NAD^+ from the SDH-NAD^+-sorbitol complex (Fig. 3) prior to docking of inhibitor to the enzyme.

4. Discussion

Many drugs exert their clinical effects by inhibiting enzyme activities. Due to the involvement of SDH in the development of various secondary complications of diabetes, this enzyme is presently of interest in terms of the design of drugs that may prevent,

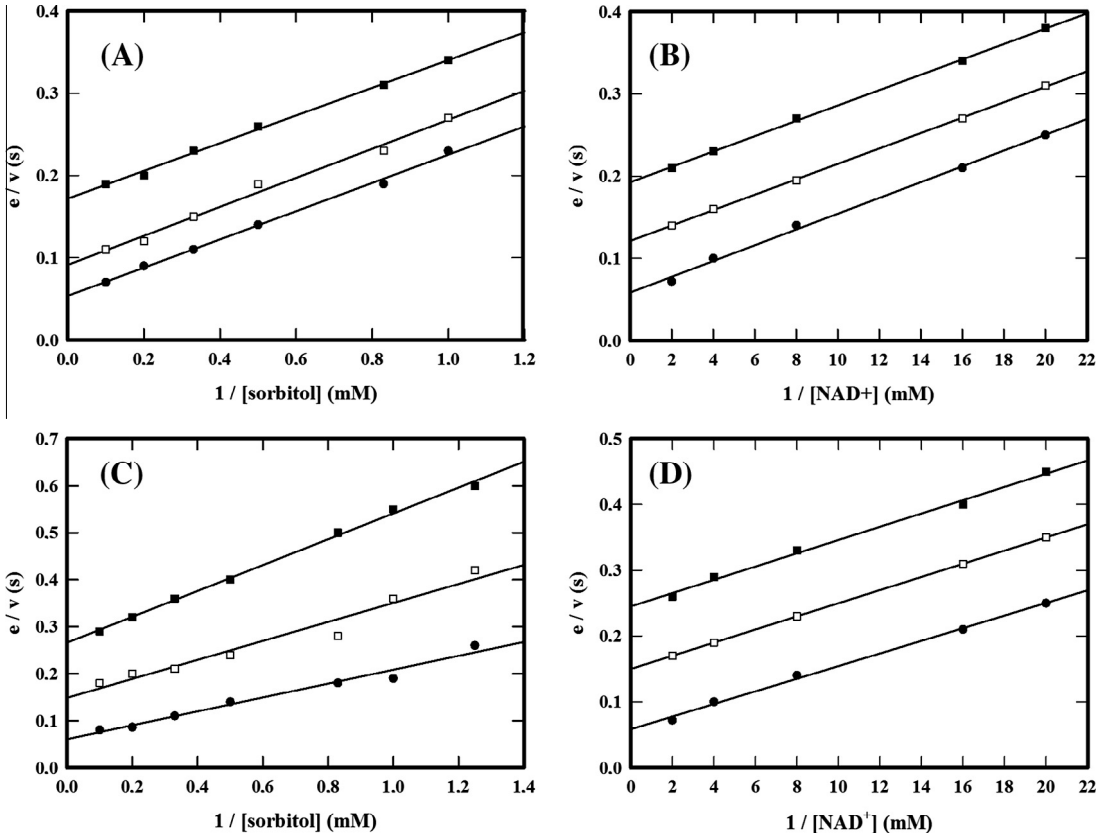

Fig. 2. Reversible inhibition of sheep liver sorbitol dehydrogenase by 2-deoxy-adenosine-5-P or Guanosine-2-P at pH 7.4. (A) Double-reciprocal plots showing the effect of 2-deoxy-adenosine-5-P on the initial rate of sorbitol oxidation at a constant saturating [NAD^+] = 500 μM. Without inhibitor (●); [inhibitor] = 1.0 mM (□), 3.0 mM (■). (B) Double-reciprocal plots showing the effect of 2-deoxy-adenosine-5-P on the initial rate of sorbitol oxidation at a constant saturating [sorbitol] = 10 mM. Without inhibitor (●); [inhibitor] = 1.0 mM (□), 3.0 mM (■). (C) Double-reciprocal plots showing the effect of Guanosine-2-P on the initial rate of sorbitol oxidation at a constant saturating [NAD^+] = 500 μM. Without inhibitor (●); [inhibitor] = 0.44 mM (□), 1.0 mM (■). (D) Double-reciprocal plots showing the effect of Guanosine-2-P on the initial rate of sorbitol oxidation at a constant saturating [sorbitol] = 10 mM. Without inhibitor (●); [inhibitor] = 0.44 mM (□), 1.0 mM (■). In each case e denotes the molar concentration of active enzyme in the assay, and v is the initial rate of the enzyme catalyzed reaction in terms of Δ [NADH] per second ($M\ s^{-1}$).

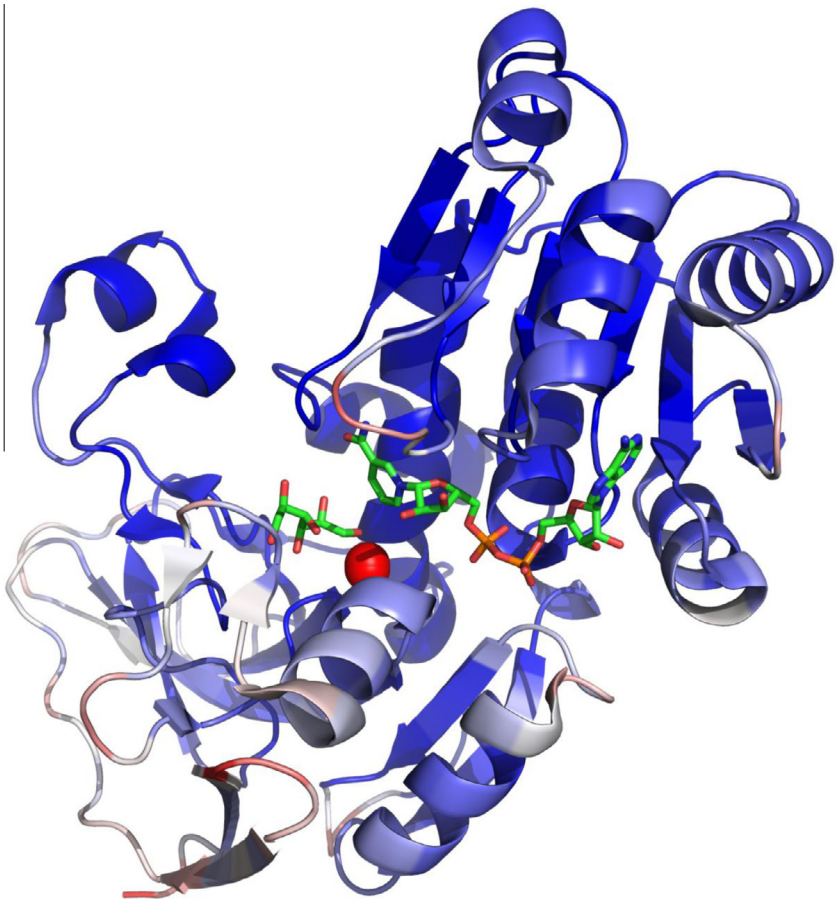

Fig. 3. The structure of the ternary complex of sheep liver sorbitol dehydrogenase with NAD^+ and sorbitol. The structure of sheep liver SDH (pdb code 3QE3) was superimposed on the structure of the human enzyme in complex with NAD^+ (pdb code 1PL8) in order to create a model of the sheep holoenzyme complex (PMDB ID code PM 0078068). The coenzyme and sorbitol molecules are shown in stick representation, omitting hydrogen atoms. Carbon, green; Oxygen, red; Nitrogen, blue; Phosphorous, yellow. The catalytic zinc is shown in red. The C-1 hydroxyl group of sorbitol is directed against the catalytic zinc, while the reactive C-2 hydroxyl group of the substrate is oriented toward the 4′-position of the nicotinamide moiety of the coenzyme. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

or alleviate, diabetic complications. Previously, various classes of compounds, including thiols, heterocyclics, carboxylic acids, amides, catecholamine derivatives and AR inhibitors, were found to inhibit sheep SDH activity *in vitro* [14,33]. It has been suggested [14] that the clinically beneficial effects of AR inhibitors on diabetic complications may partly stem from their effects on SDH activity.

Adenine nucleotides are inhibitors of various dehydrogenases, including ADH (see [27] and references cited therein) which is closely related to SDH [1,2]. In the case of various dehydrogenases cooperative effects in the presence of nucleotides have been reported, using either the purified enzymes or cell extracts [27]. The only functional form of the SDH molecule is a tetramer of identical 38-kDa subunits [1,2,34]. In the present study, however, no evidence for any cooperative effects on SDH in the presence of inhibitor was obtained.

The kinetic mechanism of sheep liver SDH is compulsory ordered with the initial binding of coenzyme to the free enzyme being a prerequisite for ternary complex formation with sorbitol [8,17] (Scheme 1).

The compulsory ordered nature of the mechanism is confirmed by studies on the bovine lens enzyme, as well as by binding studies using [^{14}C]-NAD^+ and [^{3}H]-sorbitol (see [17] and references cited therein). A conformational change in the protein structure, induced by the binding of coenzyme to the free enzyme, enables subsequent binding of substrate or inhibitor in the enzyme active site [1,5–8,35]. The interpretation of the kinetic inhibition patterns in terms of the formation of specific enzymic complexes with inhibitor is based upon the compulsory ordered nature of the kinetic mechanism of SDH [8,14,15]. In the case of uncompetitive inhibition versus sorbitol, varying the [NAD^+] using a high saturating [sorbitol] also resulted in an uncompetitive inhibition pattern (Fig. 2A and B). The lack of any slope effects in the double-reciprocal plots upon varying [NAD^+] indicates that the inhibitor does not form an enzyme-inhibitor binary complex with the enzyme. In either case, the inhibition of enzyme activity rather reflects the formation of an enzyme-NADH-inhibitor ternary complex (Scheme 1) resulting in an intercept effect in double-reciprocal plots upon varying either the concentration of sorbitol or NAD^+. Likewise, in the case of mixed inhibition with respect to sorbitol an uncompetitive inhibition pattern was seen upon varying the [NAD^+] (Fig. 2C and D). In the latter case, the intercept and slope effects reflect the formation of enzyme-NADH-inhibitor and enzyme-NAD^+-inhibitor complexes, respectively. This interpretation of the kinetic inhibition patterns is in accordance with previously published work [8,14,15,33].

It is evident that $K_{ERI} < K_{EOI}$ for virtually all compounds tested at pH 7.4. This seems to reflect the differential stability of the enzyme-NADH and enzyme-NAD^+ complexes, as the stability of the former complex increases toward lower pH [9] favoring the formation of enzyme-NADH-inhibitor complexes. For the various

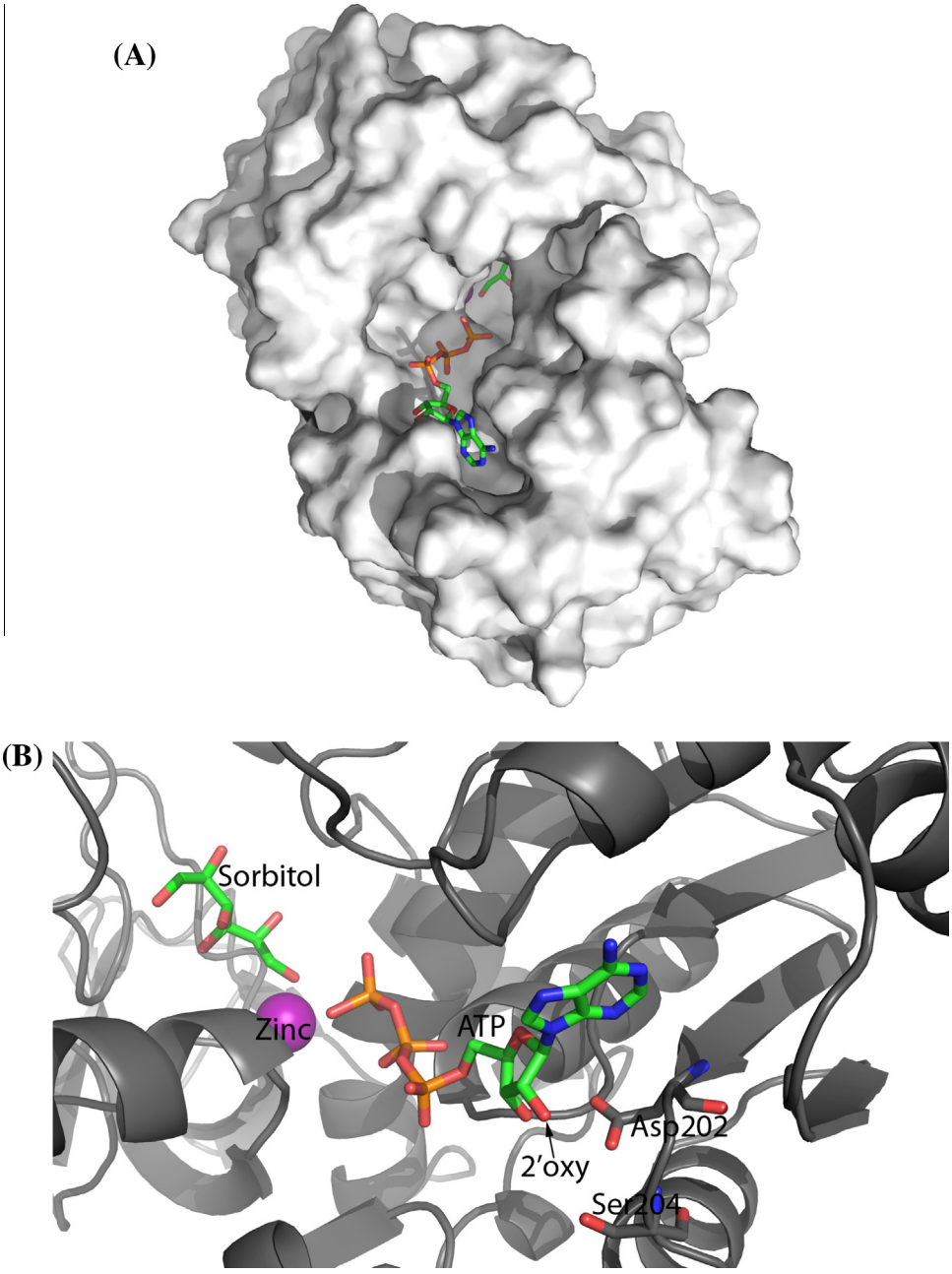

Fig. 4. ATP docked into the coenzyme binding domain of sheep liver sorbitol dehydrogenase. (A) Overall view of ATP manually docked into the active site of the ternary complex shown in Fig. 3 after removal of the NAD^+ molecule. (B) Close-up of (A).

Sorbitol	Fructose
↕	↕
$E + NAD^+ \leftrightarrow E\text{-}NAD^+ \leftrightarrow (E\text{-}NAD^+\text{-sorbitol} \leftrightarrow E\text{-}NADH\text{-fructose}) \leftrightarrow E\text{-}NADH \leftrightarrow E + NADH$	
$I \updownarrow K_{EOI}$	$K_{ERI} \updownarrow I$
$E\text{-}NAD^+\text{-}I$	E-NADH-I

Scheme 1. Kinetic inhibition mechanism of SDH.

2′-deoxy-analogues of the inhibitors used it is noticeable that the formation of such complexes is particularly favored, as indicated by the strictly uncompetitive inhibition patterns observed.

The structure of the sheep liver SDH-NAD^+-sorbitol ternary complex (Fig. 3) is in general agreement with a previous study based on manual docking of sorbitol into a homology model of human SDH [35]. There is 89% sequence homology between the human and sheep enzymes, and both the tertiary and quaternary structures are almost identical [6,7]. In the ternary complex shown in Fig. 3 the reactive C-2 hydroxyl group of sorbitol is oriented toward the 4′-position of the nicotinamide part of the coenzyme molecule, while the adjacent primary hydroxyl group binds to the catalytic zinc. Pyrimidine based SDH inhibitors bind the enzyme active site like substrate analogues via a pyrimidine linked hydroxyl group [6,17,23,35]. Specifically, in the ternary complex of NADH and SDI 158 with human SDH the pyrimidine moiety of the inhibitor interacts with the metal and the bound coenzyme [6]. The hydrophobicity of the heterocyclic structure facilitates binding. Indeed, various aromatic alcohols, including (2R)-1-phenyl-1,2-ethandiol, are substrates for mammalian SDH [11,36]. It has previously been pointed out that the nonpolar backbone of polyols, and the partly nonpolar nature of various deoxypolyol substrates of SDH, is also important for substrate binding in the enzyme active site [11].

Nucleotides are relatively weak zinc chelating agents [37]. The relatively small variations in terms of inhibitory potency between the various nucleosides and nucleotides suggest that the ribose moiety is a major determinant for the observed effects. The introduction of the 2′-deoxy arrangement resulted in a strictly uncompetitive inhibition pattern with respect to sorbitol and, specifically, the 2′-deoxy analogues of ATP, ADP and AMP exhibit similar inhibition patterns with K_{ERI} (dATP) ≈ K_{ERI} (dADP) > K_{ERI} (dAMP). The less polar 2′-deoxy-analogues binding tighter to the holoenzyme with bound NADH, in relation to that with the more polar NAD^+ molecule, suggests that the 2′-position of ribose is oriented toward the nicotinamide moiety of the coenzyme in the ternary complex, thus mimicking the interaction of sorbitol with the enzyme-NAD^+ complex (Fig. 3). Furthermore, the presence of a phosphate group in the 2′-position of ribose is positively correlated with increasing inhibitor potency. This may be interpreted in terms of the interaction of this anionic group with the NAD^+ molecule in the ternary complex. The suggestion that the cyclic and partly nonpolar compounds used in the present work interact with SDH like substrate analogues is supported by the present structure of the SDH-NAD^+-sorbitol complex, previous structure/activity studies [6,23,25,35], as well as by studies on the substrate specificity of the enzyme [11,36].

Previously, 6-thioguanosine and 6-thioinosine were found to inhibit SDH activity [14]. In both cases, the observed inhibition patterns were mixed with respect to sorbitol with the inhibitory effects being most pronounced at physiologic pH. This agrees with the present study in terms of the inhibitory patterns observed, with the effects being significantly less pronounced at the pH 9.9 optimum for sorbitol oxidation [9] than at lower pH. In the case of SDI 158 the inhibition patterns also were mixed with respect to sorbitol, and optimal inhibition of enzyme activity was seen at physiologic pH [15]. SDI 158 seems to preferentially bind the enzyme-NADH complex [15,17], as is also the case with the inhibitors used in the present study.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.04.081.

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