L-IDOSE: AN ATTRACTIVE SUBSTRATE ALTERNATIVE TO D-GLUCOSE FOR MEASURING ALDOSE REDUCTASE ACTIVITY.

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Abstract

Although glucose is one of the most important physio-pathological substrates of aldose reductase, it is not an easy molecule for in vitro investigation into the enzyme. In many cases alternative aldoses have been used for kinetic characterization and inhibition studies. However these molecules do not completely match the structural features of glucose, thus possibly leading to results that are not fully applicable to glucose. We show how aldose reductase is able to act efficiently on L-idose, the C-5 epimer of D-glucose. This is verified using both the bovine lens and the human recombinant enzymes. While the k_{cat} values obtained are essentially identical to those measured for D-glucose, a significant decrease in K_M was observed. This can be due to the significantly higher level of the free aldehyde form present in L-idose compared to D-glucose. We believe that L-idose is the best alternative to D-glucose in studies on aldose reductase.

Key words: aldose reductase; D-glucose; L-idose

Abbreviations: AR: aldose reductase; *b*AR: bovine lens aldose reductase; *h*AR: human placental recombinant aldose reductase; DTT: D,L-dithiothreitol; GAL: D,L-glyceraldehyde; IPTG: Isopropyl β-D-1-thiogalactopyranoside; S.E.M.: standard error of the mean.

INTRODUCTION

The reduction of glucose catalyzed by aldose reductase (AR), which is the first and rate-limiting step of the polyol pathway, is considered as one of the main deleterious events leading to the onset of diabetic complications. In fact, the accumulation of sorbitol and the concomitant reduction in the

antioxidant's capability are significant factors leading to cell damage (for review see:[1,2]). In addition, AR efficiently intervenes in reducing hydrophobic aldehydes, such as 4-hydroxy-2-nonenal and its Michael glutathionyl-adduct [3,4]). AR exerts a detoxifying action and at the same time triggers a cell signaling cascade related to the antioxidant defense.

Despite being claimed as an *in vivo* substrate of AR, D-glucose is far from an ideal substrate for the enzyme. In fact, despite displaying a k_{cat} comparable with other physiological and not physiological substrates [5-7], glucose is very poorly recognized by the enzyme with a measured K_M ranging between 35 and 212 mM [5, 8-12]. Nevertheless, the poor kinetic performance of the enzyme acting on glucose does not compromise the postulated involvement of AR in the induction of long-term diabetic complications. This is because in hyperglycemic conditions, time is a factor that inexorably impacts on cell damage.

Glyceraldehyde has been used in many instances as a model substrate for aldoses in *in vitro* studies on AR; however, due to the structural differences with glucose, it is advisable to go deep inside the enzyme kinetic properties using glucose itself as substrate. The kinetic data on glucose reduction are strongly affected by the hemiacetal structure of the sugar. The concentration of glucose free aldehyde, which is conceivably the form that is susceptible to the enzyme action, is very low. Its value at 25°C varies around 1.18×10^{-3} and 1.38×10^{-3} % of the total glucose concentration, as found from a CD/NMR approach and from urazole modification kinetic measurements, respectively [13]. This is mainly due to the special stability of the six membered hemiacetal ring structure of glucose, in which all the sterically significant substituents (i.e. the hydroxyl groups at C_2, C_3 and C_4 carbon atoms and the hydroxymethyl group at C_6 carbon atom) can be simultaneously allocated in an equatorial position.

The rather low level of the free aldehyde form has enabled researchers to speculate on the presumed extraordinary ability of AR to recognize glucose (i.e. its open form) as substrate [14,15]. This supported the idea of a multi-task nature of the active site of the enzyme, which is able to efficiently act on structurally different substrates, such as hydrophilic aldoses and hydrophobic aldehydes (i.e. alkanals or alkenals) [6]. In any event, the rather low level of the free aldehyde in glucose solutions creates complications for the kinetic characterization of the enzyme that acts on it. Thus, either a high concentration of the enzyme or enormous concentrations of glucose are required in order to have acceptable rate measurements. This may be critical when comparative inhibition studies are performed with different substrates [16].

L-idose is an aldo-hexose identical to D-glucose, except for the configuration at the C_5 carbon atom. This appears to have a marked effect on the stability of the hemiacetal ring in which, in the most stable chair conformation, the three hydroxyl groups at C_2, C_3 and C_4 are in an axial position and

the hydroxymethyl group at C_6 is in an equatorial position, or vice versa. The consequence is that for L-idose, the free aldehyde form (ranging from 96.9 x 10^{-3}to78.6 x 10^{-3}%, according to CD/NMR or kinetic measurements, respectively) is present in solution at a level that is approximately 60 to 80 fold higher than glucose [13]. Thus L-idose is structurally very similar to glucose, but with the advantage of exposing a concentration of the free aldehyde form that is much more suitable for enzyme activity measurements.
We propose L-idose as a new suitable substrate for the measurement of AR activity, as it mimics glucose and confirms the multi-task nature of the AR site.

Materials and Methods

Materials

L-idose came from Carbosynth. D,L-dithiothreitol (DTT), D,L-glyceraldehyde (GAL), D-glucose, Isopropyl β-D-1-thiogalactopyranoside (IPTG), NADPH, TRI® Reagent were from Sigma Aldrich. Reverse Transcriptase was from Invitrogen. dNTP and *Thermus aquaticus* DNA polymerase were from GE-Healthcare. pGEM vector was from Promega. The expression vector pET30 was from Novagen. Plasmid MiniPrep Kit was from Euroclone. The QIAEX II Extraction kit was from Qiagen. All other chemicals were of reagent grade.

Assay of aldose reductase. The activity of AR was determined at 37°C following the decrease in absorbance at 340 nm due to NADPH oxidation (ε_{340} = 6.22 $mM^{-1}\cdot cm^{-1}$). The standard assay mixture contained 0.25 M sodium phosphate buffer pH 6.8, 0.18 mM NADPH, 2.4 M ammonium sulphate, 0.5 mM EDTA and 4.7 mM GAL. One unit of enzyme activity is the amount that catalyzes the conversion of 1 μmol of substrate/min in the above assay conditions. The above assay conditions were adopted also when D-glucose or L-idose were used as substrates instead of GAL.

Expression of human recombinant aldose reductase.

Total RNA was extracted from human placenta with TRI® Reagent, following the manufacturer's protocol. cDNA was prepared from total RNA by reverse transcription, using 200 units of SuperScriptTM III Reverse Transcriptase and 0.5 μg of an oligo-dTprimer in a 50 μL total volume. The mixture also contained 0.5 mM of each dNTP, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT and 0.1 mg/mL BSA in 50 mM Tris-HCl, pH 8.3. The reaction mixture was incubated at 50°C for 60 min and the product was directly used for PCR amplification or stored at -20°C. Aliquots of 1 μL of crude cDNA were amplified in a Bio-Rad Gene CyclerTM thermocycler, using 2.5 units of *Thermus aquaticus* DNA polymerase, 1 mM of each dNTP, 1 μM of each PCR primer, 50 mMKCl,

2.5 mM $MgCl_2$ and 0.1 $mgmL^{-1}$BSA in 10 mM Tris-HCl, pH 8.3, containing 0.1% (v/v) Triton X-100. At the 5' end, we used the specific primer: 5'- CAT ATG GCA AGC CGT CTC CTG CTC AA-3', corresponding to the sequence encoding the first six amino acids of the mature protein and containing an Nde I restriction site for ligation into the expression vector, which at the same time provided the ATG codon for an additional methionine in position 1. At the 3' end the specific primer: 5'- GAA TTC TCA AAA CTC TTC ATG GAA -3' encoded the last six amino acids, followed by a stop codon and an Eco RI restriction site.

After an initial denaturation step at 95°C for 5 min, 35 amplification cycles were performed (1 min at 95°C, 30 sec at 50°C, 1 min at 72°C) followed by a final step of 7 min at 72°C. An amplification product of about 900 bp was obtained, in agreement with the expected size (948 bp). The crude PCR product was ligated into a pGEM vector without further purification, using a 1:5 (plasmid:insert) molar ratio and incubating the mixture overnight, at room temperature. After transformation of *E. coli* XL-1 Blue competent cells with the ligation product, positive colonies were selected by PCR using the plasmid's primers SP6 and T7 and grown in LB/ampicillin medium. DNA was extracted using the Plasmid MiniPrep Kit and custom sequenced at Eurofins MWG. pGEM plasmid containing the appropriate sequence was digested with Nde I and Eco RI restriction enzymes for two hours at 37°C and the digestion product was separated on agarose gel. The obtained fragment was purified from gel using QIAEX II Extraction kit and ligated into the expression vector pET30, previously linearized with the same enzymes. The resulting plasmid was sequenced and shown to encode the mature protein.

For expression of recombinant protein, the pET-30 vector, containing the sequence encoding human aldose reductase, was used to transform BL21(DE3)pLysS *E. coli* cells. Protein expression was induced by adding IPTG to a final concentration of 0.4 mM when the culture had reached a value of $O.D._{600}$ = 0.8. Cells were grown overnight at 37°C, then harvested by centrifugation and sonicated. After centrifugation, the expressed protein was obtained in soluble form. The recombinant protein presented an additional methionine at the N-terminus as the sole modification with reference to the native enzyme.

Purification of recombinant and bovine lens AR. Both the human recombinant (*h*AR) and the bovine lens AR (*b*AR) were purified following the same procedure [17]. The purity of both enzyme preparations was assessed by SDS-PAGE [18] and gels were stained with silver nitrate [19]. The specific activity of purified *b*AR and *h*AR was 1.2 and 5.3 U/mg, respectively. The purified *b*AR and *h*AR were stored at -80 °C in 10 mM sodium phosphate bufferpH 7.0 containing 2 mM DTT

alone or in the presence of 30% (w/v) glycerol, respectively. Both enzymes were extensively dialyzed against 10 mM sodium phosphate buffer pH 7.0 before use.

Other methods. Protein concentration was determined according to Bradford [20]. Statistical analysis was performed using GraphPad 3.0.

RESULTS AND DISCUSSION

Glucose reduction through the polyol pathway is considered as the physio-pathological connection between hyperglycaemic conditions and the development of long-term diabetes complications, which explains the marked interest of the research community in characterizing AR and its susceptibility to inhibition. Since glucose has rather poor features as an AR substrate, it is not easily manageable as a substrate for kinetic studies. Glyceraldehyde is mostly commonly used as a hydrophilic substrate to study AR. However glyceraldehyde does not possess one of the main features of sugars with more than three carbon atoms, i.e. the potential to generate cyclic hemiacetals. Our data show that L-idose, which is a molecule structurally very closely related to D-glucose, is a suitable substrate for measuring aldose reductase activity in kinetic as well as inhibition studies on the enzyme.

The effectiveness of L-idose as a substrate for AR was tested on purified preparations of the bovine lens and on the human placental recombinant enzymes. The usefulness of L-idose as an AR substrate is clearly evident in Fig. 1, which reports the reaction rates measured at different concentrations of *h*AR using either L-idose or D-glucose as substrates. The sensitivity of the assay using L-idose led to reliable measurements at a rather low substrate concentration, in a wide range of enzyme levels.

The reaction rates of *b*AR and *h*AR as a function of L-idose concentration are reported as double reciprocal plots in Figs. 2A and 2B, respectively. As a comparison, the same figures also show the rates of D-glucose reduction. The kinetic parameters deriving from these data are reported in Table1. Essentially the same k_{cat} values were obtained for the transformation of the two aldoses catalyzed by *b*AR. In contrast, a decrease of approximately 90 fold was observed in the K_M value, when L-idose was used as substrate rather than D-glucose. Similar considerations can be drawn for *h*AR. In fact, although the absolute values of kinetic parameters are different from those measured for *b*AR, there are essentially no differencesin the k_{cat} values for the two aldoses. A decrease of

approximately 50 fold was observed in the K_M value when L-idose was used as a substrate instead of D-glucose.

Thus the k_{cat} values measured for *h*AR confirm that L-idose and D-glucose are transformed by the enzyme through an efficient and very similar catalytic event. The K_M results could in principle be explained by the possible different abilities of both *b*AR and *h*AR to recognize the two substrates. However, the very similar structure of L-idose and D-glucose makes such an interpretation less feasible. A more useful explanation of the difference in K_M for the two substrates is the lower concentration of glucose free aldehyde compared to L-idose. In fact, on the assumption that the open chain of the substrate present at equilibrium is the only form interacting with the enzyme and using the relative concentration for both substrates that can be evaluated for the free aldehyde form in solution (approximately 1.28×10^{-3} % for D-glucose and 87.75×10^{-3} % for L-idose), calculated as the average between CD/NMR and urazole titration values [13], the emerging K_M values for D-glucose and for L-idose become much more similar than those obtained from nominal concentration values. For *b*AR, K_M values for D-glucose and L-idose of 0.9 µM and 0.7 µM, respectively, were calculated. For *h*AR, K_M values for D-glucose and L-idose of 2.6 µM and 3.5 µM, respectively, were calculated. Thus, the K_M ratios for D-glucose and L-idose obtained when nominal substrate concentrations were used (Table 1) drop to approximately 1.3 and 0.7 for *b*AR and *h*AR, respectively, when the actual free aldehyde concentration is considered.

The conclusion is that D-glucose and L-idose free aldehydes, as expected, behave similarly as AR substrates, displaying a high apparent affinity for the enzyme. Incidentally, the emergingK_M values are even lower than those reported for aldoses free aldehydes (i.e. methylglyoxal or glyceraldehyde), for which K_M values ranging between 5 and 42 µM have been reported [4,8,21-23]. It is difficult at this point to comment on the absolute values of these derived K_M for L-idose and D-glucose. In fact, the possible involvement of the hemiacetal structures in the kinetic process and a possible effect of the assay conditions on the level of the free aldehyde of both sugars could clearly affect the results.

Concerning the susceptibility to inhibition, L-idose and D-glucose reduction appear to be inhibited in the same fashion by Sorbinil, a classical inhibitor of the enzyme. The double reciprocal plots analysis of the inhibition of the *h*AR-catalyzed reaction by Sorbinil, which acts through a uncompetitive type of inhibition [9,24,25], are reported in Fig.3. The secondary plots related to the two substrates show (Fig. 3, insets) comparable values of K_i (0,88 ± 0.18 S.E.M. and 0.75 ± 0.07 S.E.M. µM for D-glucose and L-idose, respectively).

In conclusion, our results indicate L-idose as an attractive alternative substrate to D-glucose for measuring AR activity. In fact, while the two molecules are very similar in terms of structural rearrangement, L-idose offers a much more suitable concentration of the free aldehyde form for activity measurements. Our data also confirm the ability of the enzyme to act efficiently on both highly hydrophilic as well as highly hydrophobic substrates, which is a key issue to be clarified for successfully converting an aldose reductase inhibitor into an antagonizing drug for diabetic complications.

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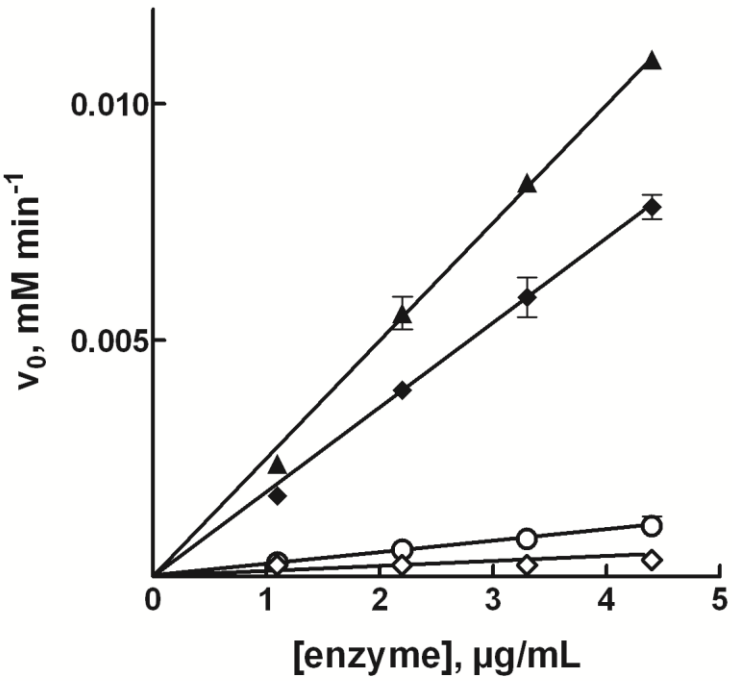
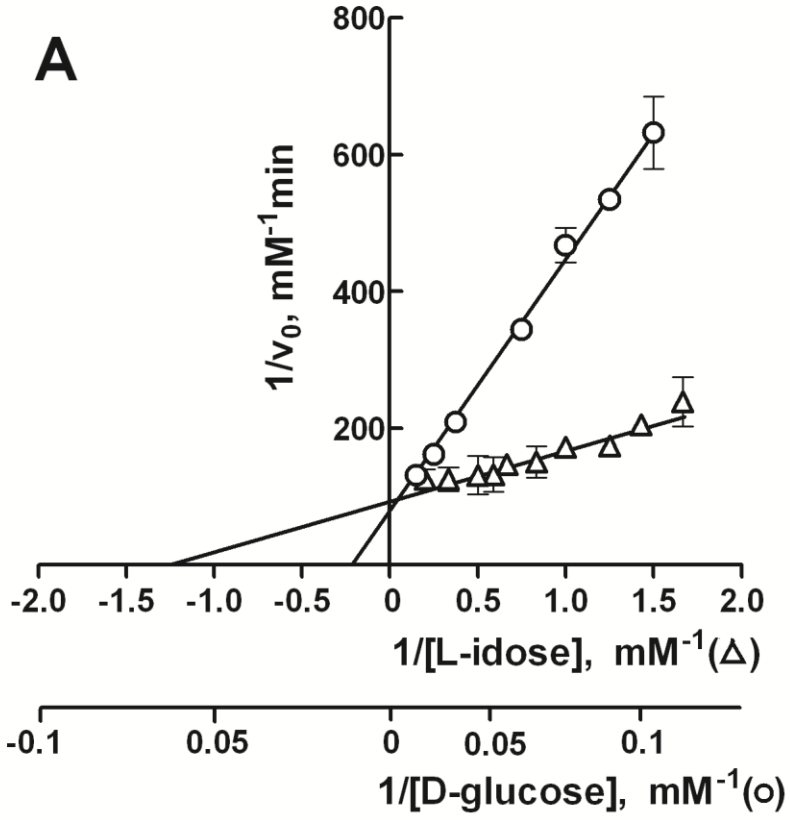


Figure 1. Evaluation of aldose reductase activity using D-glucose or L-idose as substrate.
The initial rate was evaluated at different *h*AR concentrations using the following concentrations of L-idose (closed symbols) and D-glucose (open symbols)as substrates: diamond, 2 mM; triangles, 4 mM; circles, 10 mM. Error bars (when not visible are within the symbols size) represent the standard deviations of the mean from at least three independent measurements.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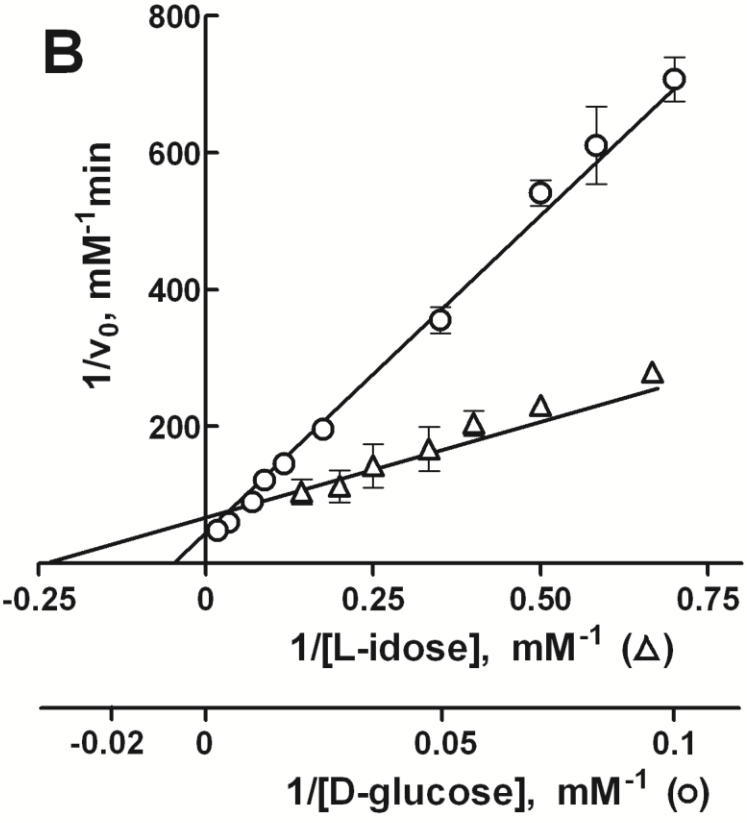
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Figure 2. Double reciprocal plots for the reduction of D-glucose and L-idose catalyzed by aldose reductase.

The initial rate measurements of the reduction of D-glucose (circles) and L-idose (triangles) are reported as double reciprocal plots. Panel A: The assays were performed in the presence of *b*AR at a final concentration of 20 mU/mL and 14 mU/mL for D-glucose and L-idose, respectively. Panel B: The assays were performed in the presence of *h*AR at a final concentration of 53 mU/mL and 24 mU/mL for D-glucose and L-idose, respectively. Error bars (when not visible are within the symbols size) represent the standard deviations of the mean from at least three independent measurements

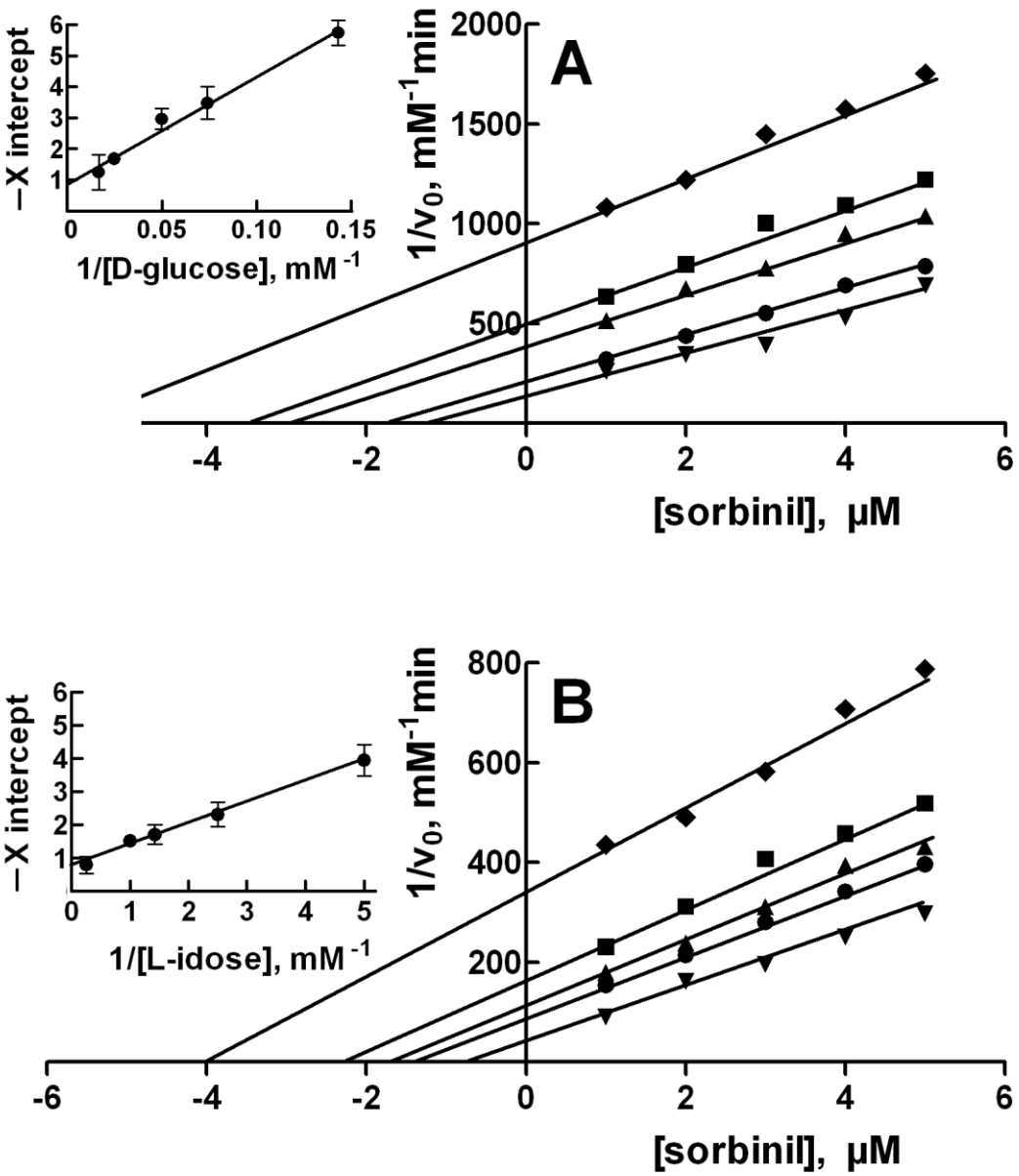

Figure 3. Inhibitory effect of sorbinil on the reduction of D-glucose and L-idose

The initial rate measurements of the reduction of D-glucose (Panel A) and L-idose (Panel B) catalyzed by *h*AR in the presence of Sorbinil are reported as Dixon plots. Insets in both panels refer

to the secondary plots of the abscissa intercept values ± S.E.M. of Dixon plots (-x intercepts) as a function of substrate concentration.