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COMPARISON OF ALDOSE REDUCTASE INHIBITORS *IN VITRO*

EFFECTS OF ENZYME PURIFICATION AND SUBSTRATE TYPE

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Abstract—Aldose reductase (EC 1.1.1.21) was purified approximately 5000-fold from bovine lens by ammonium sulphate fractionation and chromatography on DEAE-Sephacel and Matrex OA. Inhibition of this enzyme was found to depend upon the assay substrate. Tested against the purest form of enzyme, the inhibitor Sorbinil gave IC_{50} values of approximately 100 μM with the model substrate 4-nitrobenzaldehyde (4NB) and 0.4–1.4 μM with the physiological substrate glucose. A similar effect of substrate was found for the inhibitor Statil (IC_{50} 450–750 nM with 4NB, 26–71 nM with glucose substrate). The implications of these results towards the assessment of aldose reductase inhibitors *in vitro* are discussed.

Aldose reductase (EC 1.1.1.21; ALR2 [1, 2]), a member of the aldehyde reductase (ALR) family of monomeric aldo/keto reductases, has a broad substrate specificity which includes 4-nitrobenzaldehyde (4NB) as well as aldo-sugars such as glucose, galactose and xylose. Glucose is believed to be a substrate in diabetes, as the corresponding product, sorbitol, accumulates in tissues containing the enzyme [3–6]. This accumulation can be reduced or prevented by aldose reductase inhibitors (A.R.I.s), including Sorbinil [7–9] and Statil [10], which presently are in clinical trials against complications secondary to long-term diabetes mellitus [4–6].

Evaluation of A.R.I.s *in vitro* is usually carried out with an aldehyde substrate other than glucose, as high rates of reaction can be obtained more readily with low substrate concentrations and with less enzyme. D,L-glyceraldehyde (GA) is used frequently (see Table 1), although this choice has attracted criticism [11]. 4NB is used often by enzymologists as it is a substrate for all three aldehyde reductases [2].

Purification of aldose reductase (ALR2) from a variety of sources has been associated with large decreases in its sensitivity to inhibitors; as seen in Table 1, Sorbinil concentrations of at least 10 μM are required to achieve 30–60% inhibition of homogeneous ALR2. This contrasts with the much lower concentrations required for 50% inhibition of activity (IC_{50}-values) in crude preparations (0.4 μM [12], 0.15–0.5 μM [13, 14]). Furthermore, this level of susceptibility of purified enzyme *in vitro* seems poor in relation to the effectiveness of Sorbinil *in vivo*. In diabetic man, an average plasma Sorbinil concentration of 27 μM is more than sufficient to normalize erythrocyte sorbitol levels [15]. In diabetic rats, 50% reversal of sorbitol accumulation is obtained with just 0.5–1.0 mg Sorbinil/kg/day [16].

The present study was carried out to see if purification of bovine lens aldose reductase affected its sensitivity to inhibitors, and to assess whether the choice of *in vitro* substrate might contribute towards the disparity between the effectiveness of Sorbinil *in vivo* versus *in vitro*.

MATERIALS AND METHODS

Eyes from healthy bovine livestock were obtained within 6 hr of enucleation. Lenses were stored frozen for up to 4 weeks at −20°.

Matrex dye-ligand chromatography media and YM-10 ultrafiltration membranes were purchased from Amicon Ltd., Stonehouse, U.K. 4-nitrobenzaldehyde (Aldrich Chemical Co. Ltd., Gillingham, U.K.) was recrystallized from water before use. Other materials were as described previously [17].

Inhibitors. Statil (ICI 128,436), Sorbinil (CP-45,634) and M79175 were gifts from their respective manufacturers [17]. M7HEQ ((mono-7-0-β-hydroxyethyl)quercetin) originated from Zyma (Macclesfield, U.K.). Stock solutions (7.5 mM) of these compounds were prepared daily as described [17] and diluted with water. Vitamin K_1 (2-methyl-3-phytyl-1,4-naphthoquinone) and menadione (vitamin K_3; 2-methyl-1,4-naphthoquinone) purchased from Sigma Chemical Co. Ltd. (Poole, U.K.) were dissolved initially in methanol. Fructose-1-phosphate (F-1-P), fructose-1,6-bisphosphate (F-D-P), 3-phosphoglycerate (3-PG) and 2,3-bisphosphoglycerate (2,3-DPG) were purchased as cyclohexylammonium salts (Boehringer Corporation (London) Ltd., Lewes, U.K.) and dissolved in water.

Buffers. Buffer A for homogenization of lenses contained triethanolamine–HCl buffer (10 mM), $MgCl_2$ (10 mM), benzamidine hydrochloride (760 μM) and soybean trypsin inhibitor (0.2 g/l); 2-mercaptoethanol (2 mM) was added after degassing the buffer under reduced pressure. Buffer B con-

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Table 1. Reported inhibition of aldose reductases by Sorbinil *in vitro*

Enzyme source	Purification state	IC_{50} or % inhibition	Substrate used	Reference
Bovine lens	Matrex OA	approx. 100 μM		
		(26% at 10 μM)	4NB	
		0.4–1.4 μM	glucose	
	DEAE	0.5–1.5 μM	4NB	Results
		0.88 μM	glucose	
	40–70% $(NH_4)_2SO_4$/G-25	5.7–14.6 μM	4NB	
		0.4 μM	glucose	
	75%$(NH_4)_2SO_4$	0.15–0.5 μM	GA/Li	13, 14
	Crude extract	0.4 μM	GA/Li	12
Human lens	DEAE	10–20 μM†	GA/Li	22
	25–60% $(NH_4)_2SO_4$/G-75	0.2 μM	GA	32
	Crude extract	>10 μM	GA/Li	33
		0.2 μM	GA/(Li?)	31
Rabbit lens	Electrofocused	0.11 μM	GA	19
Pig brain	Essentially homogeneous	36% at 10 μM	4NB	23, 24
		53% at 10 μM	xylose	
Human brain	DEAE	6–20 μM†	GA/Li	22
		9–25 μM†	glucose/Li	
		approx. 200 μM‡	GA	34
Bovine retina	DEAE	2 μM	4NB	17
Human retinoblastoma	Crude extract	2.1 μM	GA	35
Pig muscle	Homogeneous	approx. 10 μM	GA	36
Chicken muscle	Homogeneous	35% at 10 μM	GA	21
Human muscle	DEAE	12–26 μM†	GA/Li	22
		6–20 μM†	glucose/Li	
Human erythrocytes	Homogeneous	approx. 10 μM	GA/Li	37
Human placenta	No significant inhibition with Sorbinil until selectivity eluted or electrofocused.		GA	20
	30–50% $(NH_4)_2SO_4$	0.68 μM	GA	31

* 4NB = 4-nitrobenzaldehyde; GA = D,L-glyceraldehyde; Li = assay contained approx. 0.4 M Li_2SO_4.
† Values are approximate, derived from Fig. 5 of [22] and vary with treatment of the enzyme.
‡ Value is approximate, derived from Fig. 3 of [34].

tained triethanolamine–HCl buffer (10 mM), $MgCl_2$ (10 mM) and 2-mercaptoethanol (1 mM) after degassing. Buffers A and B were pH 7.5 at 21°.

Enzyme assays. Fractions from DEAE-Sephacel were screened for aldehyde reducing activity as described previously [17]. Fractions from Matrex dye-ligand media, and enzyme pools at each purification stage, were assayed with 4NB (0.5 mM) or glucose (0.1 M) as substrate, as described previously [17] but in the absence of Li_2SO_4, azide and bovine serum albumin.

Protein assay. Protein concentration was measured by the method of Bradford [18], using turkey egg albumin as standard.

IC_{50} *determinations*. The concentration of inhibitor required to produce 50% inhibition of 0.5 mU of enzyme activity (21°) with 4NB (0.5 mM) or glucose (0.1 M) as substrate was determined in sodium–potassium phosphate buffer (0.1 M) containing NADPH (150 μM) and 2-mercaptoethanol (1 μM). Enzyme was pre-incubated with buffer containing 2-mercaptoethanol for 15–20 min; NADPH was added and the decrease in Abs. 340 nm in the presence of inhibitor was determined over 15 min; substrate was added and the decrease in Abs. 340 nm was determined over a second 15 min period. The substrate-dependent decrease was calculated and corrected for solvent blanks. Inhibitor concentrations were tested in triplicate or duplicate; variation between replicates in this assay system is low [17]. The %-remaining activity was plotted against $\log_{10}$ [inhibitor] and IC_{50} values calculated by linear regression. In the absence of added enzyme, the apparent 4NB-dependent decrease in Absorbance 340 nm was <0.001 per 15 min; thus, no significant auto-oxidation of 4NB occurred in these assay conditions.

Enzyme purification. Nuclear regions of frozen lenses were removed using a cork borer and discarded to increase the specific activity of the remaining lens material. This was homogenized in 2 vol. of Buffer A containing phenylmethyl sulphonyl fluoride (1 mM final), fractionated with ammonium sulphate (40–75% cut) as described for bovine retina [17], then desalted on Sephadex-G25. This Desalt-stage enzyme was purified partially by ion-exchange chromatography on DEAE-Sephacel as described for retina [17]; fractions containing significant ALR2 activity (4NB) were pooled and concentrated approx. 4-fold by pressure dialysis (YM-10) to produce DEAE-stage enzyme. Further purification was accomplished on Matrex Orange A (lot JH-1818, 3.4 mg dye/ml gel) which had been regenerated overnight with urea (8 M), washed extensively with Buffer B then brought to 4°. DEAE-stage enzyme was drained slowly into a Matrex OA column (16 mm i.d., 19 or 28 ml), washed with Buffer B (total addition approx. 30% column volume), then equilibrated at zero flow for 45 min. Unbound material was eluted with Buffer B at 20 cm/hr until Abs. 285 nm reached baseline. Bound material was eluted with a linear gradient of K^+ generated from Buffer B and Buffer B containing KCl (1 M). This generates

Table 2. Summary of purification of ALR2 from bovine lens*

Purification stage	Volume (ml)	Protein (mg/ml)	Activity† (mU/ml)	Specific activity (mU/mg)	Purification factor
Centrifuged homogenate	112	360	26	0.07	"1"
Desalted $(NH_4)_2SO_4$ fraction	15	145	75	0.52	7
DEAE	2.6	118	185	1.57	22
Matrex OA	1.9	0.408	150	367‡	5070

* Bow regions from 60 lenses. Percentage recovery was not calculated as aliquots of each stage were retained.

† 4NB substrate, approx. 22°.

‡ Assay 5 days later gave 220 mU/ml, 540 mU/mg protein.

a pH gradient also. Polyethylene glycol (average m.w. 1000; 10 mg/ml final conc.) was added to fractions to stabilize the enzyme; it did not affect the detection of enzyme activity. Consecutive fractions containing >15 mU activity (4NB substrate) were pooled if the specific activity exceeded 100 mU/mg protein then concentrated approx. 11-fold. This preparation, termed Matrex-stage, was used immediately for inhibition studies or stored for a few days at 4° in the presence of azide.

RESULTS

Purification

This was accomplished using the methods described for bovine retina ALR2 [17], as far as the DEAE-stage. Lens ALR2 eluted from the DEAE-Sephacel column in 0.18 M K^+. There was no evidence of ALR1 activity in this tissue by the criteria described previously [17]. Chromatography on Matrex OA was the most effective purification step (Table 2). The enzyme was eluted from Matrex OA in 0.15–0.2 M K^+. The 5000-fold purification reported in Table 2 is relative to lens material that had been dissected to increase its specific activity, and is therefore an underestimate of the purification relative to intact lenses. The specific activity of ALR2 at the Matrex-stage exceeded 0.2 U/mg protein (4NB substrate) but this increased 50–70% on storage at 4° for a few days. This increase is similar to that occurring with bovine kidney ALR2 (C. Grimshaw, Aldose Reductase Workshop, Hawaii, U.S.A., 1984; personal communication, 1986). The final ratio of specific activity with 4NB to that with glucose was 10.9 and 10.8 in two separate preparations of lens ALR2.

Studies with known inhibitors

At the earliest stage of purification (centrifuged lens homogenate, buffer-exchanged on Sephadex-G25) aldose reductase was more susceptible to the designed inhibitors Statil, Sorbinil or M79175 when glucose was the substrate, compared with 4NB (Table 3). No such effect was seen with the less specific inhibitor M7HEQ.

A similar dependence of inhibition upon the choice of substrate was found with enzyme obtained by ammonium sulphate fractionation (desalted completely on Sephadex-G25). Sorbinil was more effective when tested against glucose (IC_{50} 0.40 μM) than 4NB (IC_{50} 5.7–14.6 μM) as substrate. The effect was seen with Statil also (IC_{50} 0.14 μM vs glucose; 0.32 μM vs 4NB).

Table 3. Inhibition of crude* bovine lens ALR2 with glucose or 4-nitrobenzaldehyde (4NB) as substrate

Inhibitor	% Inhibition†	
	Glucose	4NB
Statil	100 (100)	40 (60)
Sorbinil	75 (95)	20 (55)
M79175	105 (100)	55 (70)
M7HEQ	0 (30)	10 (30)

* High speed supernate buffer-exchanged on Sephadex G-25.

† Values are means of 2 or 3 replicates, given to nearest 5%, for 1 μM (10 μM) inhibitor.

Further purification by ion-exchange chromatography did not increase the susceptibility of the enzyme with glucose as substrate to inhibition by either Sorbinil or Statil (IC_{50} values 0.88 μM and 0.19 μM, respectively). However, this DEAE-stage enzyme preparation was more sensitive to these inhibitors when assayed with 4NB (IC_{50} values: 0.5–1.5 μM for Sorbinil; 0.05 μM for Statil).

After the final purification step (dye-ligand chromatography on Matrex OA) inhibition was, as before, greater with glucose as substrate than with 4NB. This effect (Fig. 1) occurred with Sorbinil and with Statil in each of two enzyme preparations (IC_{50} value prep. 1 (prep. 2): Sorbinil 0.41 μM (1.4 μM) vs glucose, approx. 100 μM (approx. 100 μM) vs 4NB; Statil 26 nM (71 nM) vs glucose, 450 nM (750 nM) vs 4NB). In complete contrast, M7HEQ gave greater inhibition of 4NB-dependent activity than that with glucose (70% vs 30% inhibition by 10 μM M7HEQ). The designed inhibitor M79175 displayed the same substrate dependence of inhibition seen with Statil and Sorbinil. It always gave slightly more inhibition than its close structural analogue Sorbinil, in agreement with a previous report of their relative effectiveness *in vitro* [19].

The substantial decrease in the sensitivity of ALR2 to inhibition (using 4NB as substrate) which occurred at the final purification stage was not an artefact resulting from the difference in specific activity, *per se*, between Matrex-stage and DEAE-stage enzyme, as increasing the protein conc. of Matrex-stage

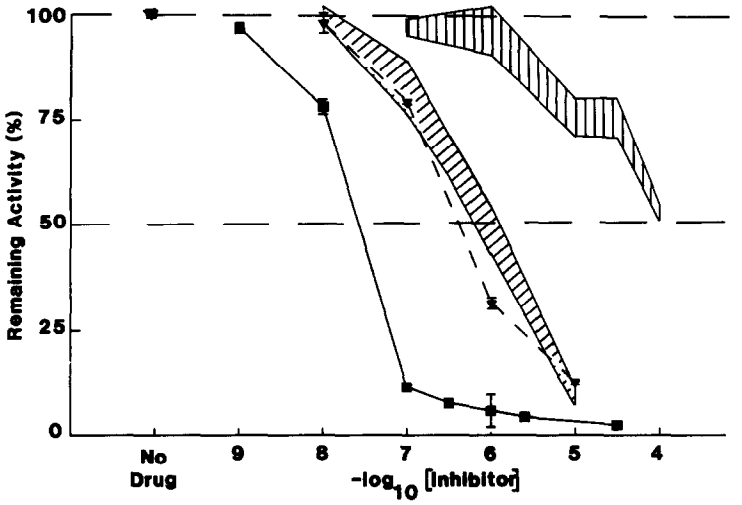

Fig. 1. Inhibition of Matrex-stage ALR2 by Statil and Sorbinil with glucose or 4-nitrobenzaldehyde (4NB) as substrate. –■– Statil vs glucose (two IC_{50} determinations). ▨ Statil vs 4NB; range of values ± ovalbumin or ultrafiltrate (see text). –▼– Sorbinil vs. glucose. ▥ Sorbinil vs. 4NB; range of values ± ovalbumin. Replicates are within bar limits.

enzyme to that of DEAE-stage by adding ovalbumin (120 mg/ml final) did not alter its IC_{50} values. The addition of a 10-fold excess of DEAE-stage ultrafiltrate to Matrex-stage enzyme did not reduce the IC_{50} values. Thus, no evidence was obtained for the presence of a stable endogenous modulator of this enzyme in bovine lens, in contrast to certain other tissues [20].

Studies with putative inhibitors

Neither vitamin K (K_1) nor menadione (K_3) were substrates, or inhibitors, of the purest form of bovine lens ALR2. Menadione is, however, an inhibitor of ALR2 from bovine retina [17] and chicken muscle [21], in addition to being a substrate for ALR3 [2].

The phosphorylated sugars F-1-P, F-D-P, 3-PG and 2,3-DPG (at 15 μM) were tested as inhibitors of Desalt-stage and Matrex-stage enzyme with glucose or 4NB as substrate. Neither F-1-P nor F-D-P had significant effects. 3-PG gave moderate inhibition (10% Desalt-stage, 5% Matrex-stage), but only with 4NB as substrate. 2,3-DPG inhibited activity with 4NB moderately (8% Desalt-stage, 7% Matrex-stage) but inhibited activity with glucose only at the Matrex-stage (8% inhibition). 3-PG and 2,3-DPG are inhibitors of partially purified human ALR2 [22].

DISCUSSION

Substantial purification of bovine lens aldose reductase produced a form of the enzyme that was remarkably insensitive (IC_{50} approx. 100 μM) to inhibition by Sorbinil and its analogue M79175 when the model substrate 4NB was used. However, the IC_{50} values obtained with the identical enzyme preparations but using glucose as substrate were as low as 0.4 μM, reflecting more closely the known potency of Sorbinil *in vivo*. Thus, the previously observed insensitivity of purified ALR2 to inhibition (Introduction, Table 1) could well have been due to the choice of assay substrate.

Substrate dependence of inhibition is not unusual in cases where the inhibitor affects the binding of substrate. However, as Sorbinil [30] and Statil [10] are uncompetitive inhibitors interacting with the enzyme at a site distinct from the substrate binding pocket, an alternative explanation for the effect must be found.

Comparison of the %-inhibition produced by Sorbinil with the two substrates in circumstances when the ratios of inhibitor to enzyme were very similar (approx. 2.4:1) confirmed that Sorbinil was more effective against the enzyme with glucose (55% inhibition) than with 4NB as substrate (<5% inhibition). Similarly, Statil gave 95% inhibition of activity with glucose in comparison with 15% inhibition of activity with 4NB. Thus, the substrate dependence of inhibition was not related to the different amounts of enzyme present in assays with glucose as substrate compared with 4NB.

Flynn and Cromlish [23, 24] found a similar substrate dependence of inhibition in their studies of homogeneous ALR2 from pig brain. They used xylose and 4NB as substrates and the effect was noted for Sorbinil and Alrestatin (another designed inhibitor) but not for the less specific inhibitor Quercetin (related to M7HEQ). Similar effects have been noted for ALR2 from human retina [25].

The model substrate GA interacts with only a part of the substrate binding pocket of ALR2 utilized by glucose [26, 27]. This difference may account for the fact that the kinetic mechanism employed by pure bovine lens ALR2 with GA substrate differs from that with glucose or galactose as substrate [28]. Similarly, the kinetics of bovine kidney ALR2 with 4NB or pyridine-3-al as substrate are different from those with glucose, xylose, arabinose or ribose as substrate [29].

One possible explanation for these observations is that interaction of glucose with regions of the substrate binding pocket inaccessible to model

substrates, better preserves the characteristics of the inhibitor binding site.

The results above show that the choice of substrate influences considerably the interaction of inhibitors with the enzyme. This suggests that the characteristics of the inhibitor binding site with model substrates may differ significantly from those present with glucose as substrate, as in diabetes. Yet, it is common to use a model substrate *in vitro* in studies of inhibitor–structure/activity relationships directed towards the design of novel inhibitors for use against diabetic complications [e.g. 7, 19, 30, 31]. The use of glucose as substrate in such studies would seem to be advisable.

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