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(2,6-DIMETHYLPHENYLSULPHONYL)NITROMETHANE: A NEW STRUCTURAL TYPE OF ALDOSE REDUCTASE INHIBITOR WHICH FOLLOWS BIPHASIC KINETICS AND USES AN ALLOSTERIC BINDING SITE

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Abstract—Many of the complications of diabetes seem to be due to aldose reductase (aldehyde reductase 2, ALR2) catalysing the increased conversion of glucose to sorbitol. Therapy with aldose reductase inhibitors (ARIs) could, therefore, decrease the development of diabetic complications. (2,6-Dimethylphenylsulphonyl)nitromethane (ICI 215918) is an example from a newly discovered class of ARIs, and we here describe its kinetic properties. Preparations of bovine lens ALR2 exhibit biphasic kinetics with respect to glucose and various inhibitors including ICI 215918. The inhibitor sensitive form (ALR2S) has a higher affinity for glucose than does the inhibitor insensitive form (ALR2I). Only ALR2S was characterized in detail because ALR2I activity is very low at physiological levels of glucose and is difficult to measure with accuracy. Aldehyde reductase (ALR1) is the most closely related enzyme to ALR2. Inhibition of ALR1 was, therefore, investigated in order to assess the specificity of ICI 215918. The values of K_i and K_{ies} (dissociation constants for inhibitor from enzyme–inhibitor and enzyme–inhibitor–substrate complexes, respectively) for ICI 215918 with bovine kidney ALR1 and bovine lens ALR2S have been determined. When glucose is varied, the compound is an uncompetitive inhibitor of ALR2S ($K_{ies} = 0.10\ \mu M$ and K_i is $\gg K_{ies}$), indicating that ICI 215918 associates with an allosteric site on the enzyme. These kinetic characteristics would cause a decrease in the concentration required to give 50% inhibition when glucose levels rise during hyperglycaemia. ICI 215918 is a mixed noncompetitive inhibitor of ALR1 ($K_i = 10\ \mu M$ and $K_{ies} = 1.8\ \mu M$) when glucuronate is varied. Thus, the compound has up to 100-fold specificity in favour of ALR2S relative to ALR1. Therapeutic interest has now centred upon at least three distinct structural types of ARIs: spirohydantoins, acetic acids and sulphonylnitromethanes. Using one representative of each type, we have demonstrated kinetic competition for inhibition of ALR2S. This observation strongly suggests that the different inhibitors use overlapping binding sites.

Aldose reductase (aldehyde reductase 2, ALR2†) (EC 1.1.1.21) is strongly associated with the pathogenesis of some symptoms of diabetes [1, 2]. The enzyme catalyses reduction of the open-chain, aldehyde form of glucose to sorbitol [3] and there is evidence suggesting that excessive flux leads to damage in nerve, kidney, retina and lens. Resultant mechanisms of tissue damage (see Ref. 2) appear to include: depletion of NADPH, decreased myoinositol, altered Na^+,K^+-ATPase activity and cytoplasmic accumulation of sorbitol and its metabolite, fructose, which in turn leads to hypertonicity and osmotic uptake of water. Accordingly, inhibition of ALR2 represents a potential therapeutic approach to reducing the development of diabetic complications [2, 4].

Spirohydantoins and acetic acids were the first ARIs to be evaluated for clinical potential (see Ref. 2). The inhibitor now under investigation, ICI 215918 (Fig. 1), is an example of a new structural type of ARI, the sulphonylnitromethanes. This compound has been selected for detailed kinetic study because it exhibits promising biological properties when administered to diabetic animals. We, therefore, now present the first characterization of the kinetics of inhibition of ALR2 by a sulphonylnitromethane.

A possible problem which could arise during therapy with ARIs is specificity for the target enzyme. This is a particular concern because ALR2 is closely related to other members of the aldo-keto reductase family [1, 5–8]. Aldehyde reductase (aldehyde reductase 1, ALR1) (EC 1.1.1.2) exhibits the highest homology in function and structure. There is 51% identity in amino acid sequence between human ALR1 and ALR2 [8]. We have, therefore, also characterized the kinetics of inhibition of bovine kidney ALR1 by ICI 215918.

MATERIALS AND METHODS

Materials

Reagents were purchased from the Sigma Chemical Co. (Poole, U.K.) and BDH (Poole, U.K.). Bovine kidney ALR1 and bovine lens ALR2 were prepared as described previously [9] and were generous gifts from S. Freeman and C. M. Sennitt. ARIs were synthesized at ICI Pharmaceuticals (see Ref. 10).

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† Abbreviations: ALR1, aldehyde reductase 1, aldehyde reductase; ALR2, aldehyde reductase 2, aldose reductase; ALR2I, ALR2 which is insensitive to inhibitors; ALR2S, ALR2 which is sensitive to inhibitors; ARI, aldose reductase inhibitor; ICI 215918, (2,6-dimethylphenylsulphonyl)nitromethane; I, inhibitor; S, substrate; E, enzyme.

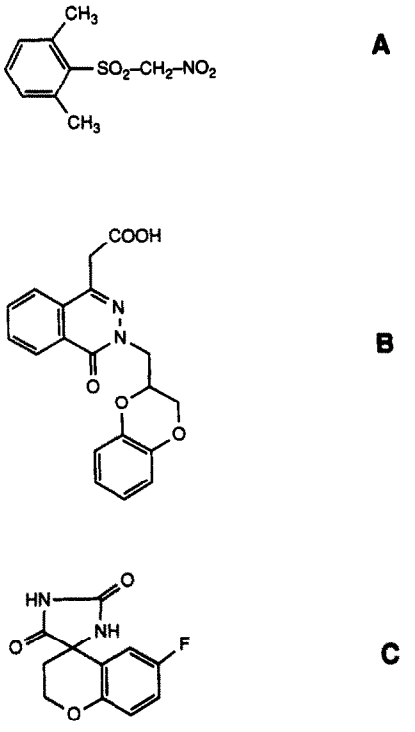

Fig. 1. Structures of inhibitors used in this study: (A) ICI 215918, (2,6-dimethylphenylsulphonyl)nitromethane; (B) Compound I, 2-(2*H*,3*H*-benzodioxin-2-ylmethyl)-1*H*,2*H*-1-oxophthalazin-4-ylacetic acid; (C) sorbinil, *d*-6-fluoro-spiro-(chroman-4,4′-imidazolidine)-2′,5′-dione.

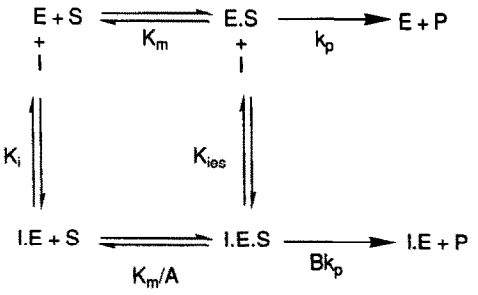

Fig. 2. General kinetic mechanism for reversible enzyme inhibition. Details are given in Methods.

Methods

Assay of ALR1 and ALR2. ALR1 activity was measured as described previously [9]. The standard assay conditions for ALR2 were as follows: 64 mM 3-(*N*-morpholino)propanesulphonic acid/NaOH, pH 7.0, 0.1 mM EDTA, 100 μM NADPH, 50 mM D-glucose and 10 μL dimethyl sulphoxide per 1 cm^3 assay. Reagents were incubated at 25° and the assay was started by addition of enzyme (to approximately 1 mg protein/cm^3). Rate was followed spectrophotometrically at 340 nm, taking the extinction coefficient of NADPH as 6200 M^{-1} cm^{-1}.

Measurement of substrate specificity and inhibitor sensitivity demonstrated no detectable contamination of the ALR2 preparation with ALR1 and vice versa (see Ref. 9). Both ALR1 and ALR2 displayed values of K_m for NADPH of <5 μM. Each enzyme was, therefore, saturated with respect to NADPH whenever there was sufficient coenzyme to follow the rate by measuring E_{340}.

All inhibitors followed reversible kinetics because a good fit was obtained using rate equations for reversible inhibition, and preincubation of enzyme with inhibitor prior to addition of substrate gave the same rate as when assays were started by addition of enzyme.

Measurement of protein concentration. The Folin method was used.

Identification of kinetic mechanism

The preparations of bovine lens ALR2 follow biphasic kinetics with respect to both substrate and inhibitor (see Results). The form with a high affinity for glucose is sensitive to inhibitors and so is referred to as ALR2S. The high K_m form is insensitive to ARIs and is referred to as ALR2I.

Rate equations involving ALR1 or ALR2S. Rate in the absence of inhibitor was fitted to the Michaelis–Menten equation. The expression for substrate inhibition was taken as

$$v = V_{max}[S]/(K_m + [S] + [S]^2/K_{is}) \quad (1)$$

where K_{is} is the constant for substrate inhibition [11]. The values of K_{is} were $\gg K_m$ so that, when studying ARIs, substrate inhibition was avoided by using $[S] \ll K_{is}$. The effects of ARIs were analysed using

$$v = v_0/(1 + [I]/K_i') \quad (2)$$

where v_0 is rate in the absence of I, and K_i' is the IC_{50}. Both v_0 and K_i' vary according to [S] (see Refs. 9 and 12). For tight binding inhibitors (where [I] is not $\gg$ total enzyme concentration, $[E]_t$ (Ref. 13)), the rate equation is

$$v = v_0\{-0.5(K_i'/[E]_t + [I]/[E]_t - 1) + 0.5\sqrt{((K_i'/[E]_t + [I]/[E]_t - 1)^2 + 4K_i'/[E]_t)}\}. \quad (3)$$

The kinetics of inhibitors were analysed using the general scheme shown in Fig. 2 where k_p is the rate constant for conversion of E.S complex to E + P, A and B are constants, and K_m, K_i and K_{ies} are dissociation constants (K_i and K_{ies} are dissociation constants for I from IE and IES complexes, respectively). The system was characterized by determining the values of K_m, k_p, K_i, K_{ies}, A, B and $[E]_t$. In each case, several of these parameters were redundant because the mechanism was less complex than that illustrated. Data were, therefore, analysed by fitting of different rate equations which were derived from Fig. 2 by taking various parameters to be redundant (see Table 1).

Rate equations involving ALR2I. Substrate dependence was analysed simply by adding a second Michaelis–Menten expression in order to represent the contribution from ALR2I. Similarly, biphasic inhibition at constant [S] was analysed by adding Eqn 2 to either Eqn 2 or 3, depending on whether or not the sensitive form followed tight binding

Table 1. Mechanisms of enzyme inhibition used for analysis of kinetic data

Mechanism	Simplifying assumptions			
	K_i	K_{ies}	B	$[E]_t$
MNC tight binding			0	
PNC tight binding		$= K_i$	0	
UC tight binding	$\gg K_{ies}$		0	
C tight binding		$\gg K_i$	0	
Partial MNC				$\ll [I]$
Partial PNC		$= K_i$		$\ll [I]$
Partial UC	$\gg K_{ies}$			$\ll [I]$
Partial C		$\gg K_i$	1	$\ll [I]$
Mixed noncompetitive			0	$\ll [I]$
Pure noncompetitive		$= K_i$	0	$\ll [I]$
Uncompetitive	$\gg K_{ies}$		0	$\ll [I]$
Competitive		$\gg K_i$	0	$\ll [I]$

Measured rates were analysed by fitting of 12 rate equations which describe different mechanisms. The mechanisms are derived by making assumptions to simplify a complex scheme (Fig. 2) which often contains redundant parameters (see Methods). MNC, mixed noncompetitive; PNC, pure noncompetitive; UC, uncompetitive; C, competitive. The rate equations are given in Refs 11–13.

kinetics. Biphasic inhibition when [S] was varied followed relationships which were constructed by adding the rate equation for the sensitive form to the expression $[S](V^I_{max}/K^I_m)$. This procedure assumed that: (a) there was no significant inhibition of the insensitive form and (b) the [S] was $\ll K^I_m$. These assumptions were consistent with the measured rates (see Results).

Fitting of kinetic data. Measured initial velocities were analysed using the rate equations for different mechanisms. Fitting was performed by unweighted non-linear regression using the secant algorithm in the NLIN procedure of SAS [14]. When substrate and inhibitor, or two different inhibitors, were varied in the same experiment, rates were analysed by multivariate regression in a single fit.

The kinetic mechanism each data set was identified as follows. An F-test (see Refs 15–17) was used to compare the residual sum of squares following fitting to several different rate equations in order to identify the most suitable mechanism, i.e. the one with the largest number of fitting parameters, none of which was redundant. This test estimated the probability, P, that the improvement in fit associated with inclusion of the extra parameter arose due to chance. Thus, the term was justified by the data if P was low (<1%), but not if it was high (>5%). The following criteria were used to assess the quality of the fit for the selected equation. The parameter values and SEs had to be reasonable, and the residual differences between observed and calculated rates had to be small and show a random distribution [16, 18].

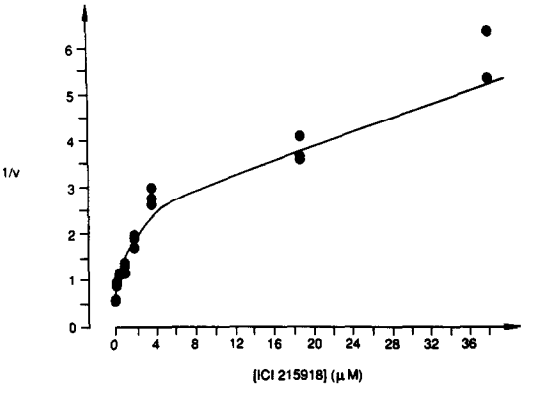

Fig. 3. Inhibition of ALR2 by ICI 215918 at 167 mM D-glucose. Assays were performed as described in Methods. The best fit line is shown on a Dixon plot [19], and was calculated as described in Methods. The IC_{50} values for ALR2S and ALR2I were 0.23 ± 0.04 and $30 \pm 20\,\mu M$, respectively, with 77% of the total activity being due to ALR2S. Rate was measured in nmol NADPH utilized/min/mg protein.

RESULTS

The aldose reductase preparation exhibits biphasic kinetics

A number of publications demonstrate that ALR2 from various sources follows biphasic kinetics (see Discussion). We also see this behaviour on a Dixon plot [19] for the new sulphonylnitromethane, ICI 215918 (Fig. 3), and representatives from each of two other structural types of ARI, the spirohydantoin, sorbinil, and the acetic acid, ponalrestat (Table 2). Ponalrestat and sorbinil were investigated because they are amongst the best studied ARIs. We find that many other compounds from each of these three structural types also follow biphasic kinetics where 60–100% of the enzyme activity is sensitive to the inhibitor. The sensitive form was denoted ALR2S and the insensitive form was named ALR2I. IC_{50} values for ALR2I are difficult to measure with precision because this form constitutes only a minor fraction of the total enzyme activity. ALR1 does not utilize glucose (see Refs 6 and 9). Thus, ALR2I

Table 2. IC_{50} values for different ARIs

Structural type	Compound	IC_{50} for ALR2S (μM)	IC_{50} for ALR2I (μM)
Spirohydantoin	Sorbinil	0.29 ± 0.02	500 ± 300
Acetic acid	Ponalrestat	0.0049 ± 0.0005	5 ± 3
Sulphonylnitromethane	ICI 215918	0.23 ± 0.04	30 ± 20

Assays were performed as described in Ward *et al.* [9]. D-Glucose (167 mM) was used as substrate. Rate in the absence of inhibitor was 3.0 nmol/min/mg protein and ALR2S accounted for about 90% of the activity. The best fit values are quoted ± SE. Inhibitor concentrations were varied from at least low $IC_{50}/5$ to high IC_{50}. The structures of ICI 215918 and sorbinil are shown in Fig. 1, and that for ponalrestat is given by Ward *et al.* [9].

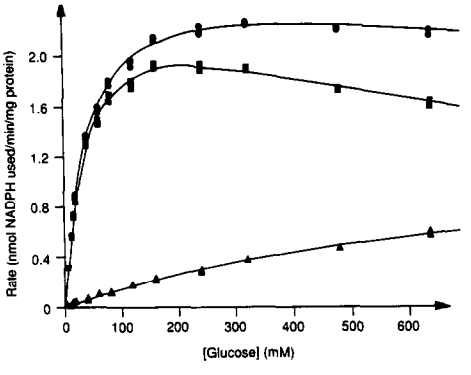

Fig. 4. Glucose dependence of ALR2I and ALR2S. (●) total ALR2 activity; (▲) ALR2I; (■) ALR2S. Assays were performed in the presence or absence of 0.4 μM ponalrestat as described by Ward *et al.* [9]. ALR2I and ALR2S activities were calculated as detailed in Results. The best fit lines are shown and were determined using the techniques given in Methods. Note that high concentrations of glucose led to a decrease in ALR2S activity. Data were, therefore, fitted to a relationship which includes substrate inhibition of ALR2S (Eqn 1, see Methods) with K_{is} = 1200 ± 100 mM. V_{max} = 2.6 ± 0.1 and 1.2 ± 0.1 nmol/min/mg protein for ALR2S and ALR2I, respectively.

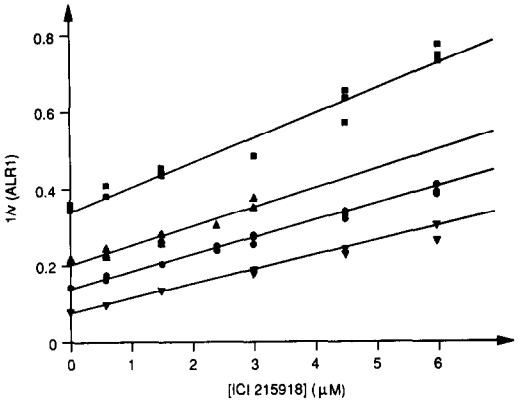

Fig. 5. Plot for inhibition of ALR1 by ICI 215918. Assays and data analysis were performed as described in Methods. The best fit lines are shown on a Dixon plot [19] and these were calculated assuming mixed noncompetitive inhibition (see text). (▼) 7.17 μM; (●) 1.02 μM; (▲) 0.57 μM; (■) 0.29 μM D-glucuronate. Data relating to 1.43 and 4.92 μM D-glucuronate have been omitted for clarity. Rate was measured in nmol NADPH utilized/min/mg protein.

activity cannot be explained by contamination of the ALR2 preparation with ALR1.

There is a large difference between the IC_{50} values against the two kinetic forms (Table 2) such that an intermediate concentration can be used to fully inhibit ALR2S whilst having an undetectable effect on ALR2I. This approach was used to characterize the glucose dependence (Fig. 4). The velocity in the absence of inhibitor is due to both ALR2S and ALR2I. Only ALR2I is resistant to 0.4 μM ponalrestat and the rate due to ALR2S is calculated by subtracting rate in the presence of the inhibitor from the total velocity. The calculated K_m values of ALR2S and ALR2I for glucose are 40 ± 1 and 800 ± 50 mM, respectively. Validity of the calculated K_m for ALR2I is dependent upon the inhibitor not affecting the affinity for glucose. This condition is likely to be valid because ponalrestat does not affect the K_m of ALR2S [9]. Furthermore, the K_m values calculated using this approach are similar to those reported previously for sensitive and insensitive forms of ALR2 [9, 20–23].

Inhibition of ALR1 by ICI 215918

D-Glucuronate was used as the substrate and data were analysed as described in Methods. Figure 5 is a Dixon plot which illustrates the data together with the calculated best fit lines. These results show that the sulphonylnitromethane leads to mixed noncompetitive inhibition of ALR1 as it follows the rate equation

$$v = \frac{V_{max}[S]/(1 + [I]/K_{ies})}{[S] + K_m(1 + [I]/K_i)/(1 + [I]/K_{ies})} \quad (4)$$

where V_{max} = 15.2 ± 0.2 nmol/min/mg protein, K_m = 1.2 ± 0.1 mM, K_i = 10 ± 2 μM and K_{ies} = 1.8 ± 0.1 μM. The value of K_m is close to that of 1.3 mM determined for ALR1 from ox kidney [5]. The calculated kinetic parameters indicate that inhibition tends towards being uncompetitive because K_i is greater than K_{ies}. Uncompetitive inhibition (where the K_i term is redundant, see Table 1) is rejected because there is a large improvement in the

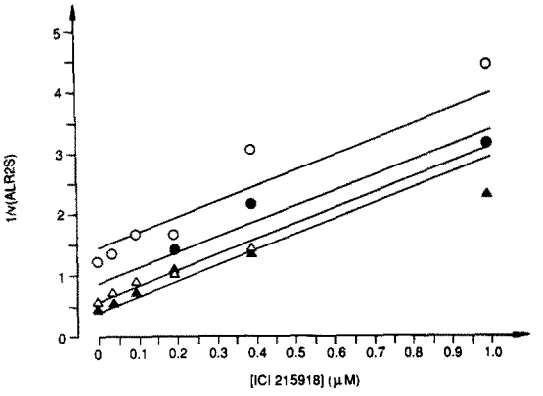

Fig. 6. Plot for inhibition of ALR2S by ICI 215918. Assays and data analysis were performed as described in Methods. The best fit lines are shown on a Dixon plot [19] and these were calculated assuming uncompetitive inhibition (see text). (▲) 64 mM; (△) 16 mM; (●) 8 mM; (○) 4 mM D-glucose. In order to aid clarity, values are plotted as the means of duplicates which agreed to within ±15% and data relating to 32 mM D-glucose have been omitted. Rate was measured in nmol NADPH utilized/min/mg protein.

fit ($P < 0.1\%$) after inclusion of the K_i parameter to give mixed noncompetitive inhibition (see Methods).

Inhibition of ALR2S by ICI 215918

D-glucose was used as the substrate and data were analysed as described in Methods. The highest glucose concentration used was 64 mM. This value was selected because it is a sufficient multiple of K_m (=18 mM for these data) to allow determination of K_{ies}, but is low enough to avoid substrate inhibition (K_{is} = 1200 mM). The measured K_m of 18 mM is close to that of 13 mM obtained for ALR2S from bovine kidney [22]. The values of kinetic parameters were calculated by fitting to Eqn 5 and the plot in Fig. 6 was constructed by calculating the rate due to ALR2S as total rate minus that due to ALR2I. Thus, there is a large variation in these data which is amplified at low rates by taking $1/v$ for the Dixon plot. Nevertheless, the results do clearly show that the sulphonylnitromethane leads to uncompetitive inhibition of ALR2S and so follows the rate equation

$$v = \frac{V_{max}[S]/(1 + [I]/K_{ies})}{[S] + K_m/(1 + [I]/K_{ies})} + \frac{V^I_{max}}{K^I_m}[S] \quad (5)$$

where $V_{max} = 3.8 \pm 0.2$ nmol/min/mg protein, $K_m = 18 \pm 2$ mM, and $K_{ies} = 0.10 \pm 0.01$ μM. The V^I_{max}/K^I_m term relates to the activity of ALR2I which is not affected by these concentrations of ICI 215918. The values of V^I_{max} and K^I_m could not be separated because [S] is $\ll K^I_m$. The best fit value for V^I_{max}/K^I_m is 0.004 ± 0.001 nmol/min/mM/mg protein. Mixed noncompetitive inhibition is rejected because there is not a large improvement in the fit ($P \simeq 20\%$) after inclusion of the K_i parameter (see Table 1 and Methods).

Inhibition of ALR2S by mixtures of compounds

Rates were measured in the presence of various concentrations of two different inhibitors and analysed by fitting to the relationship

$$v = \{v_0/(1 + [I]/K'_i + [X]/K'_x + \beta[I][X]/K'_iK'_x)\} + v^I \quad (6)$$

where v_0 is rate in the absence of inhibitors. K'_i (the inhibition constant at fixed substrate concentration) and K'_x are the IC_{50} values for the two inhibitors, I and X, and v^I is the rate due to ALR2I. The independence term for mixtures of inhibitors, β, is equal to zero when I and X use the same site. If I and X use different sites, then $\beta = 1$. A value of $\beta > 1$ indicates that binding of one inhibitor favours association with the second, whereas $0 < \beta < 1$ shows that prior association with one compound decreases affinity for the second. β is the inverse of the Yonetani and Theorell interaction constant, α [24]. One advantage of using β is that, if the inhibitors compete for the same site, its value tends to zero, making it into a redundant parameter which can be rejected using an F-test (see Methods). In this case, Eqn 6 becomes

$$v = \{v_0/(1 + [I]/K'_i + [X]/K'_x)\} + v^I. \quad (7)$$

At least three different structural types of ARI are now potential therapeutic agents: spirohydantoins, acetic acids and sulphonylnitromethanes. Experiments have been performed using three pairwise combinations of one representative from each type (sorbinil, Compound I and ICI 215918, respectively). Compound I (structure in Fig. 1) replaced ponalrestat as the acetic acid in these experiments because kinetic analysis using ponalrestat is complicated by its being a tight binding inhibitor of ALR2S [9]. Occurrence of tight binding kinetics introduces an additional parameter ($[E]_t$) into the rate equations and so makes quantitative analysis more difficult. When I and X use the same binding site, parallel lines are obtained on Yonetani–Theorell plots. Conversely, the lines intersect on the abscissa at $[I] = -K'_i$ if I and X bind independently [24]. This qualitative analysis suggests that the three inhibitors share overlapping binding sites on ALR2S (Fig. 7), a conclusion which is confirmed by calculation of β values (Table 3). Each pairwise combination gives values for β which are very close to zero. The possibility that inhibitor binding is independent (β = 1) is clearly disproved by this analysis. An F-ratio (see Methods) was used to test for redundancy of β in Eqn 6 and the value of P was >20% in each case, indicating that Eqn 7 best described the data.

DISCUSSION

Biphasic kinetics for ALR2

Several groups have demonstrated the existence of forms of aldose reductase which differ in their affinity for substrate and inhibitors (for example see Refs 22, 23, 25–32). Such biphasic kinetics may be difficult to detect in some systems because one phase is obscured by the slow formation of an inhibitory enzyme-bound complex containing aldehyde and NADPH [33]. Our data extend the observation of biphasic kinetics by demonstrating two forms with different sensitivities to a new structural type of

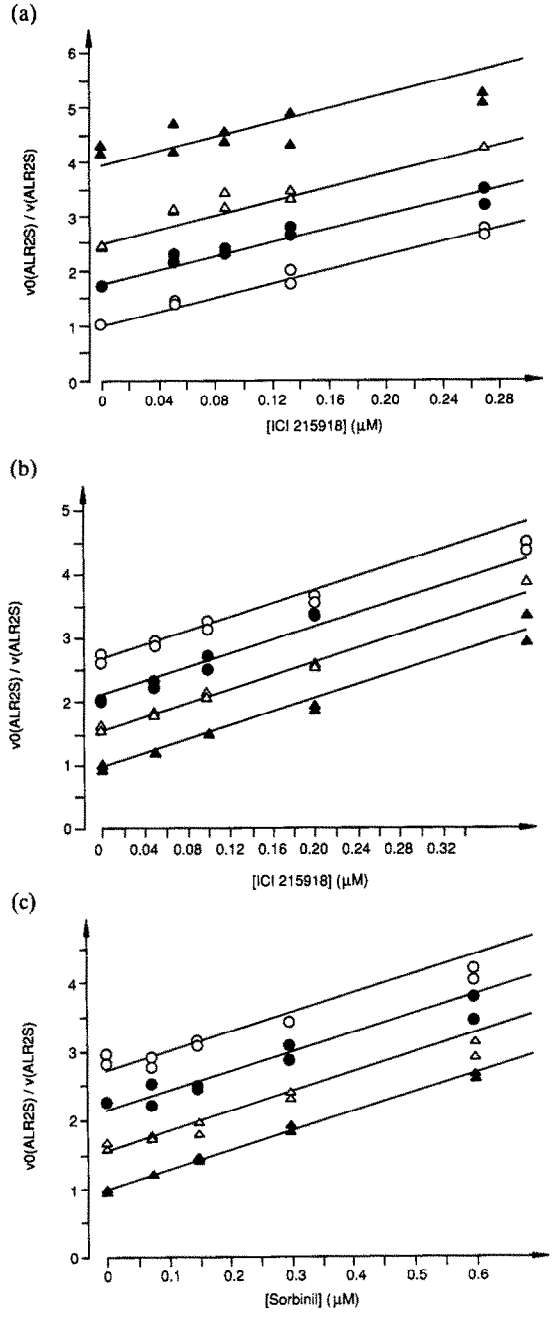

Fig. 7. Yonetani–Theorell plots for ALR2S. Assays and data analysis were performed as described in Methods. D-glucose was fixed at 100 mM and inhibitors were varied from around 0.3 to 5 times IC_{50}. The best fit lines are shown and these were calculated assuming that both inhibitors use the same binding site (see text). Data relating to some inhibitor concentrations have been omitted for clarity. (a) ICI 215918 and sorbinil: (○) 0 μM; (●) 0.22 μM; (△) 0.43 μM; (▲) 0.86 μM sorbinil. Calculated IC_{50} values were 0.18 ± 0.01 and 0.32 ± 0.02 μM for ICI 215918 and sorbinil, respectively. (b) ICI 215918 and Compound I: (▲) 0 μM; (△) 0.60 μM; (●) 1.20 μM; (○) 1.80 μM Compound I. Calculated IC_{50} values were 0.21 ± 0.01 and 1.18 ± 0.06 μM for ICI 215918 and Compound I, respectively. (c) Compound I and sorbinil: (▲) 0 μM; (△) 0.60 μM; (●) 1.20 μM; (○) 1.80 μM Compound I. Calculated IC_{50} values were 0.95 ± 0.05 and 0.32 ± 0.02 μM for Compound I and sorbinil, respectively.

Table 3. Inhibition of ALR2S by mixtures of ARIs

Inhibitors used		β
ICI 215918	Sorbinil	0.03 ± 0.12
ICI 215918	Compound I	0.04 ± 0.09
Compound I	Sorbinil	−0.08 ± 0.05

Assays and data analysis were performed as described in Methods and the legend for Fig. 7. D-Glucose was fixed at 100 mM. Values of β were calculated by fitting to Eqn 6 and are quoted ± SE.

inhibitor, a sulphonylnitromethane (Fig. 3). The two kinetic components in our preparation also differ markedly in affinity for other types of ARI and glucose (Table 2 and Fig. 4). The minor form, ALR2I, has a high K_m for glucose and a low affinity for inhibitors.

Autoxidation of glucose could be responsible for the second kinetic phase, especially since some ARIs can scavenge free radicals [34]. This explanation, however, seems unlikely because production of sorbitol from glucose is stoichiometrically related to oxidation of NADPH, at least in the presence of ALR2 purified from rat lens [35]. The following observations on our data suggest that ALR2I is a form of ALR2, rather than being due to autoxidation or a contaminating enzyme. ALR2I activity varies according to the glucose concentration and is affected by ARIs of different structural types. The contribution of ALR2I varies between enzyme preparations suggesting that the multiple phases may arise from modification of a single form of the enzyme during isolation, storage, or assay. ALR2I activity does not result from contamination with ALR1 because this enzyme does not use glucose [6, 9]. Further evidence indicating that preparations of bovine ALR2 contain two forms of the same enzyme comes from the studies of Grimshaw *et al.* [22] who demonstrated that homogeneous enzyme purified from bovine kidney is converted during storage from a low K_m/low V_{max} form into a high K_m/high V_{max} form. This activation process reduces the sensitivity to a range of ARIs, including spirohydantoins (sorbinil, AL 1576) and acetic acids (ponalrestat, tolrestat). The relationship between the two kinetic forms in our preparation is not clear. One form may be generated by proteolysis, binding of a regulatory molecule [27], glycosylation [28–30], oxidation [23, 31, 32, 36, 37] or a conformation change [22]. Any of these processes may account for the changes in kinetic properties which have been observed during purification of ALR2 [23, 27, 38, 39]. Alternatively, biphasic kinetics may result from the presence in bovine lens of different enzymes which catalyse glucose-dependent oxidation of NADPH. Closely related isoenzymes of ALR2 have been reported for enzyme prepared from several sources [40]. Two rather different proteins have also been reported to possess aldose reductase activity and susceptibility to ARIs. ALR2 (which is isolated from, for example, human placenta and muscle, and bovine lens) contains 20–23 Pro and 7–9 Cys residues

[8, 21, 41, 42]. The second protein (which has been isolated, for example, from human lens and erythrocyte) has only 12–15 Pro and 1–2 Cys residues [39, 43, 44]. The difference in structure and tissue distribution between these proteins is confirmed by immunological studies. Antibody against bovine kidney ALR2 binds to a protein from human lens, muscle and placenta, but does not recognise any antigens in human erythrocytes [45].

The importance of ALR2I *in vivo* is not clear, but this form is responsible for only a small fraction ($\leq$3%) of the total ALR2 activity in our assays at physiological levels of glucose ($\leq$20 mM). The contribution of ALR2I *in vivo*, however, may be much higher under certain conditions, within specific tissues or in selected individuals [27–30].

Inhibition of ALR1 and ALR2S by ICI 215918

The compound causes mixed noncompetitive inhibition of ALR1 when the glucuronate concentration is varied. The respective values of K_i and K_{ies} are 10 and 1.8 μM, so that inhibition tends towards being uncompetitive. The IC_{50}, therefore, varies according to the concentration of substrate, being high at low [S], and low at high [S]. The value of K_i sets a maximum limit to the value of IC_{50} when [S] is $\ll K_m$ and K_{ies} represents the minimum IC_{50} which occurs when [S] is $\gg K_m$. This is because prior binding of glucuronate causes an increase in the affinity of ALR1 for ICI 215918 which inhibits the enzyme primarily by preventing catalysis. The values of kinetic constants and the mechanism of inhibition could, however, be different if an alternative substrate were used. Glucuronate is one of several naturally occurring substrates of ALR1 [6], so the characteristics *in vivo* are likely to resemble those in our assay.

ICI 215918 causes uncompetitive inhibition of ALR2S when the glucose concentration is varied. The value K_{ies} is 0.10 μM whereas K_i is too high to calculate from our data. The IC_{50} again varies according to the concentration of substrate, being very high at low [S] and low at high [S]. The IC_{50} is high when [S] is $\ll K_m$ and decreases to a minimum which is equal to K_{ies} when [S] is $\gg K_m$. This is because prior binding of glucose is required for detectable association of the enzyme and inhibitor which again acts by preventing catalysis. The values of kinetic constants and the mechanism of inhibition could be different for an alternative substrate but glucose is, of course, the one which appears to be involved in generating diabetic complications.

The physiological range of glucose concentrations is typically from 3 to 20 mM. At these levels, the IC_{50} for ICI 215918 against ALR2S is between 0.24 and 0.71 μM, being lower at high glucose concentrations. Selectivity for inhibition of ALR2S rather than ALR1 varies according to substrate concentration but falls in the range $K_{ies}/0.71 = 2.5$ to $K_i/0.24 = 42$. Thus, ICI 215918 exhibits less selectivity than the acetic acid, ponalrestat (range 390–7800) [9], but has similar specificity to many other ARIs including AL1576, tolrestat and sorbinil (selectivities from 1.9 to 39 based on IC_{50} values) [46].

ICI 215918 does not compete with the binding of either glucuronate to ALR1, or glucose to ALR2S, indicating that it does not utilize the binding sites for either of these substrates. This observation correlates with the lack of structural similarity between the substrates and the inhibitor. ICI 215918 is, therefore, unlikely to bind to the substrate binding sites on other enzymes which utilize glucose or glucuronate and so is unlikely to generate side-effects via such a mechanism.

Both ALR1 and ALR2 follow a compulsory ordered kinetic mechanism where reduced coenzyme binds before the aldehyde [39, 47–50]. Any inhibitor which uses the coenzyme binding site is, therefore, likely to follow competitive kinetics with respect to aldehyde [11]. The only case where this does not hold is if the inhibitor binds to the coenzyme site but fails to interfere with association of aldehyde. ICI 215918 is, therefore, unlikely to use the coenzyme binding site on either ALR1 or ALR2S. This contention is supported by the observation that ICI 215918 seems to utilize the same binding site as other ARIs which do not compete with NADPH (see below).

Different ARIs seem to use the same binding site

The mechanism of inhibition has been characterized for a number of ARIs of different structural types. Spirohydantoins, such as sorbinil or AL1576, and acetic acids, such as tolrestat or ponalrestat, inhibit both ALR1 and ALR2 by following either a noncompetitive or uncompetitive mechanism when either aldehyde or NADPH is varied (see Refs 2, 9, 46, 51–53). We have now shown ICI 215918, which is a representative of a further structural type of ARI, the sulphonylnitromethanes, behaving similarly. These mechanisms of inhibition indicate that the compounds do not utilize the substrate or NADPH binding sites on ALR1 and ALR2. All of these data, therefore, strongly suggest the existence of a single allosteric regulatory site which binds different structural types of ARI. This hypothesis is supported by kinetic competition experiments with several acetic acids and spirohydantoins [46, 53, 54]. Molecular modelling and analysis of structure–activity relationships have been used to explain these results in terms of a single inhibitor binding site [55]. Our data (Table 3 and Fig. 7) now indicate kinetic competition between ICI 215918 and both an acetic acid and a spirohydantoin. Thus, a new type of ARI, a sulphonylnitromethane, appears to use the same binding site as spirohydantoins and acetic acids. These data could be explained by a region of the ARI binding site accommodating acidic groups such as the acetate residue of ponalrestat, the hydantoin moiety of sorbinil and the nitro group of ICI 215918.

The observation of a high affinity binding site for inhibitors on ALR2 suggests that the ARI binding site has a functional role in healthy cells. However, no naturally occurring ligands have been demonstrated to use the ARI binding site. Some studies indicate that ALR2 from different tissues exhibits a marked variation in sensitivity to several different ARIs. Placental ALR2 is typically less sensitive than lens enzyme with respect to inhibition by either spirohydantoins or acetic acids [27, 52, 56]. In one study [27], purification of human placental

ALR2 led to an increase in sensitivity to spirohydantoins. Addition of a heat-inactivated placental extract decreased the sensitivity of the purified enzyme, suggesting that molecules in the extract bind to a regulatory site which may be shared with ARIs. The regulatory factor was not identified. Elucidation of the structure of any natural ligands for the ARI binding site would provide valuable information for the design of new inhibitors and would advance understanding of the physiological role and regulation of the enzyme.

REFERENCES

1. Flynn TG, Aldehyde reductases: monomeric NADPH-dependent oxidoreductases with multifunctional potential. *Biochem Pharmacol* **31**: 2705–2712, 1982.
2. Kador PF, The role of aldose reductase in the development of diabetic complications. *Medicinal Res Rev* **8**: 325–352, 1988.
3. Inagaki K, Miwa I and Okuda J, Affinity purification and glucose specificity of aldose reductase from bovine lens. *Arch Biochem Biophys* **216**: 337–344, 1982.
4. Sima AAF, Bril V, Nathaniel V, McEwan TAJ, Brown MB, Lattimer SA and Greene DA, Regeneration and repair of myelinated fibers in sural nerve biopsy specimens from patients with diabetic neuropathy treated with sorbinil. *N Eng J Med* **319**: 548–555, 1988.
5. Daly AK and Mantle TJ, Purification and characterisation of the multiple forms of aldehyde reductase in ox kidney. *Biochem J* **205**: 373–380, 1982.
6. Cromlish JA and Flynn TG, Identification of pig brain aldehyde reductases with the high-K_m aldehyde reductase, the low-K_m aldehyde reductase and aldose reductase, carbonyl reductase, and succinic semialdehyde reductase. *J Neurochem* **44**: 1485–1493, 1985.
7. Watanabe K, Fujii Y, Nakayama K, Ohkubo H, Karamitsu S, Kagayama H, Nakanishi S and Hayaishi O, Structural similarity of bovine lung prostaglandin F synthase to ε-crystallin of the European common frog. *Proc Natl Acad Sci USA* **85**: 11–15, 1988.
8. Bohren KM, Bullock B, Wermuth B and Gabbay KH, The aldo-keto reductase superfamily. *J Biol Chem* **264**: 9547–9551, 1989.
9. Ward WHJ, Sennitt CM, Ross H, Dingle A, Timms D, Mirrless DJ and Tuffin DP, Ponalrestat: a potent and specific inhibitor of aldose reductase. *Biochem Pharmacol* **39**: 337–346, 1990.
10. Brown SP, Cooper AL, Longridge JL, Morris JJ and Preston J, (Arylsulphonyl)nitromethanes, process for their preparation and use in pharmaceutical compositions. *European Patent Application*, Publication No. 304190, 1989.
11. Segal IH, *Enzyme kinetics*. John Wiley & Sons, New York, 1975.
12. Cleland WW, Statistical analysis of enzyme kinetic data. *Methods Enzymol* **63**: 103–138, 1979.
13. Williams JW and Morrison JF, The kinetics of reversible tight binding inhibition. *Methods Enzymol* **69**: 437–467, 1979.
14. SAS, The NLIN procedure. In: *SAS User's Guide, Version 5 Edn*, pp. 575–606. SAS Institute, Cary, NC, 1985.
15. Haarhoff KN, Use of multivariate non-linear regression analysis in fitting enzyme kinetic models. *J Theor Biol* **22**: 117–150, 1969.
16. Mannervik B, Regression analysis, experimental error, and statistical criteria in the design and analysis of experiments for discrimination between rival kinetic models. *Methods Enzymol* **87**: 370–390, 1982.
17. Duggleby RG, Determination of inhibition constants, IC_{50} values and the type of inhibition for enzyme-catalysed reactions. *Biochem Med Metab Biol* **40**: 204–212, 1988.
18. Ellis KJ and Duggleby RG, What happens when data are fitted to the wrong equation? *Biochem J* **171**: 513–517, 1978.
19. Dixon M, The determination of enzyme inhibitor constants. *Biochem J* **55**: 170–171, 1953.
20. Cromlish JA and Flynn TG, Pig muscle aldose reductase. *J Biol Chem* **258**: 3583–3586, 1983.
21. Morjana NA and Flynn TG, Aldose reductase from human psoas muscle. *J Biol Chem* **264**: 2906–2911, 1989.
22. Grimshaw CE, Shahbaz M, Jahangiri G, Putney CG, McKercher SR and Mathur EJ, Kinetic and structural effects of activation of bovine kidney aldose reductase. *Biochemistry* **28**: 5343–5353, 1989.
23. Vander Jagt DL, Robinson B, Taylor KK and Hunsaker LA, Aldose reductase from human skeletal and heart muscle. *J Biol Chem* **265**: 20982–20987, 1990.
24. Yonetani T and Theorell H, Studies on liver alcohol dehydrogenase complexes. III. Multiple inhibition kinetics in the presence of two competitive inhibitors. *Arch Biochem Biophys* **106**: 243–251, 1964.
25. Sheaff CM and Doughty CC, Physical and kinetic properties of homogeneous bovine lens aldose reductase. *J Biol Chem* **251**: 2696–2702, 1976.
26. Conrad SM and Doughty CC, Comparative studies on aldose reductase from bovine, rat and human lens. *Biochim Biophys Acta* **708**: 348–357, 1982.
27. Maragoudakis ME, Wasvary J, Hankin H and Gargiulo P, Human placental aldose reductase. Forms sensitive and insensitive to inhibition by alrestatin. *Mol Pharmacol* **25**: 425–430, 1984.
28. Das B and Srivastava SK, Activation of aldose reductase from human tissues. *Diabetes* **34**: 1145–1150, 1985.
29. Srivastava SK, Hair GA and Das B, Activated and unactivated forms of human erythrocyte aldose reductase. *Proc Natl Acad Sci USA* **82**: 7222–7226, 1985.
30. Srivastava SK, Ansari NH, Hair GA, Jaspan J, Rao MB and Das B, Hyperglycaemia induced activation of human erythrocyte aldose reductase and alteration in kinetic properties. *Biochim Biophys Acta* **870**: 302–311, 1986.
31. Del Corso A, Barsacchi D, Camici M, Garland D and Mura U, Bovine lens aldose reductase: identification of two enzyme forms. *Arch Biochem Biophys* **270**: 604–610, 1989.
32. Del Corso A, Barsacchi D, Gianessi M, Tozzi MG, Camici M and Mura U, Change in stereospecificity of bovine lens aldose reductase modified by oxidative stress. *J Biol Chem* **264**: 17653–17655, 1989.
33. Grimshaw CE, Shahbaz M and Putney CG, Mechanistic basis for non-linear kinetics of aldehyde reduction catalyzed by aldose reductase. *Biochemistry* **29**: 9947–9955, 1990.
34. Wolff SP and Crabbe MJC, Low apparent aldose reductase activity produced by monosaccharide autoxidation. *Biochem J* **226**: 625–630, 1985.
35. Kador PF, Kinoshita J, Brittain DR, Mirrless DJ, Sennitt CM and Stribling D, Purified rat lens aldose reductase. Polyol production *in vitro* and its inhibition by aldose reductase inhibitors. *Biochem J* **240**: 233–237, 1986.
36. Wermuth B and Von Wartburg J-P, Aldose reductase from human tissues. *Methods Enzymol* **89**: 181–186, 1982.
37. Vander Jagt DL, Hunsaker LA, Robinson B, Stangebye LA and Deck LM, Aldehyde and aldose reductases from human placenta. Heterogeneous expression of

multiple enzyme forms. *J Biol Chem* **265**: 10912–10918, 1990.
38. Poulsom R, Comparison of aldose reductase inhibitors *in vitro*. Effects of enzyme purification and substrate type. *Biochem Pharmacol* **36**: 1577–1581, 1987.
39. Vander Jagt DL, Stangebye LA, Hunsaker LA, Eaton RP and Sibbitt WL Jr, Purification of aldose reductase from human placenta and stabilization of the inhibitor binding site. *Biochem Pharmacol* **37**: 1051–1056, 1988.
40. Cromlish JA and Flynn TG, Purification and characterisation of two aldose reductase isoenzymes from rabbit muscle. *J Biol Chem* **258**: 3416–3424, 1983.
41. Yoo H and McGuinness ET, Pig brain aldose reductase: purification, significance of amino-acid composition, substrate specificity and mechanism. *Int J Biochem* **19**: 865–871, 1987.
42. Schade SZ, Early SL, Williams TR, Kézdy FJ, Heinrickson RL, Grimshaw CE and Doughty CC, Sequence analysis of bovine lens aldose reductase. *J Biol Chem* **265**: 3628–3635, 1990.
43. Das B and Srivastava SK, Purification and properties of aldose reductase and aldehyde reductase II from human erythrocyte. *Arch Biochem Biophys* **238**: 670–679, 1985.
44. Das B, Song HP, Ansari WH, Hair GH and Srivastava SK, Purification and properties of aldose reductase and aldehyde reductase II from human lens. *Lens Res* **4**: 309–335, 1988.
45. Grimshaw CE and Mathur EJ, Immunoquantitation of aldose reductase in human tissue. *Anal Biochem* **176**: 66–71, 1989.
46. Sato S and Kador PF, Inhibition of aldehyde reductase by aldose reductase inhibitors. *Biochem Pharmacol* **40**: 1033–1042, 1990.
47. Halder AB and Crabbe MJC, Bovine lens aldehyde reductase (aldose reductase). Purification kinetics, and mechanism. *Biochem J* **219**: 33–40, 1984.
48. Davidson WS and Flynn TG, Kinetics and mechanism of aldehyde reductase from pig kidney. *Biochem J* **177**: 595–601, 1979.
49. Ryle CM and Tipton KF, Kinetic studies with the low K_m aldehyde reductase from ox brain. *Biochem J* **227**: 621–627, 1985.
50. Wermuth B, Burgisser H, Bohren K and Von Wartburg JP, Purification and characterisation of human brain aldose reductase. *Eur J Biochem* **127**: 279–284, 1982.
51. Kador PF, Goosey JD, Sharpless NE, Kolish J and Miller DD, Stereospecific inhibition of aldose reductase. *Eur J Med Chem* **16**: 293–298, 1981.
52. O'Brien MM, Schofield PJ and Edwards MR, Inhibition of human brain aldose reductase and hexonate dehydrogenase by alrestatin. *J Neurochem* **39**: 810–814, 1982.
53. Bhatnagar A, Liu S, Das B, Ansari NH and Srivastava SK, Inhibition kinetics of human kidney aldose and aldehyde reductases by aldose reductase inhibitors. *Biochem Pharmacol* **39**: 1115–1124, 1990.
54. Kador PF, Kinoshita JF and Sharpless NE, The aldose reductase inhibitor site. *Metabolism* **35** (Suppl): 109–113, 1986.
55. Kador PF and Sharpless NE, Pharmacophor requirements of the aldose reductase inhibitor site. *Mol Pharmacol* **24**: 521–531, 1983.
56. Kador PF, Kinoshita JF, Tung WH and Chylack LT, Differences in the susceptibilities of various aldose reductases to inhibition. *Invest Ophthalmol Visual Sci* **19**: 980–982, 1980.