Aldehyde reductase (ALR1) and aldose reductase (ALR2) were purified from human placenta by a rapid and efficient scheme that included rapid extraction of both reductases from 100,000×g supernatant material with Red Sepharose followed by purification by chromatofocusing on Pharmacia PBE 94 and then chromatography on a hydroxylapatite high performance liquid chromatography column. Expression of ALR1 and ALR2 in placenta is variable with ALR1/ALR2 ratios ranging from 1:4 to 4:1. ALR1 and ALR2 are immunochemically distinct. ALR1 shows broad specificity for aldehydes but does not efficiently catalyze the reduction of glucose due to poor binding (K_m = 2.5 mM). ALR1 exhibits substrate inhibition with many substrates. ALR2 also shows broad specificity for aldehydes. Although glucose is a poor substrate for ALR2 compared with other substrates, the affinity of ALR2 for glucose (K_m = 70 mM) suggests that glucose can be a substrate under hyperglycemic conditions. ALR2 shows normal hyperbolic kinetics with most substrates except with glyceraldehyde, which exhibits substrate activation. Treatment of ALR2 with dithiothreitol converted it into a form that exhibited hyperbolic kinetics with glyceraldehyde. Dithiothreitol treatment of ALR2 did not alter its properties toward other substrates or affect its inhibition by aldose reductase inhibitors such as sorbinil (2,4-dihydro-6-fluorospiro-[4H-1-benzopyran-4,4’-imidazolidine]-2’,5’-dione), tolrestat (N-[4-fluorophenyl]-1-naphthalenyl)thioxomethyl]-N-methylglycine), or statil (3-[(4-bromo-2-fluorophenyl)methyl]-3,4-dihydro-4-oxo-1-phthalazinonic acid).

NADPH-dependent oxidoreductases represent a multigene family of monomeric proteins that are widely distributed in man and animals (1-4). Although the physiological functions of these enzymes are not clear, one of their functions may be the detoxification of reactive carbonyl-containing compounds. The several members of this family of oxidoreductases are immunochemically distinct (1). One member of this family of enzymes has been variously named aldehyde reductase (EC 1.1.1.2; alcohol:NADP+ oxidoreductase), high K_m aldehyde reductase, mevaldate reductase, daunorubicin-pH 8.5 reductase, lactaldehyde reductase, glucuronate reductase, and ALR1 (2, 4-8). A second member has been named aldose reductase (EC 1.1.1.21; alditol:NADP+ oxidoreductase), low K_m aldose reductase, and ALR2 (8, 9). A third member has been called carboxylic sterase, prostaglandin 9-ketoreductase, daunorubicin-pH 6.0 reductase, and ALR3 (8, 10). In spite of these different names, all of these enzymes are capable of catalyzing the reduction of a wide range of aldehydes and ketones, some of which are xenobiotics and others of which are endogenous compounds.

ALR2 has received special attention recently due to its possible role in the reduction of glucose during diabetic hyperglycemia (11, 12). ALR2 and sorbitol dehydrogenase form the polyol pathway by which glucose is reduced to sorbitol and sorbitol is oxidized to fructose (13). In most tissues, this is normally a minor pathway for glucose metabolism. During hyperglycemia, this pathway increases, especially in tissues with insulin-independent uptake of glucose (14). Sorbitol levels may increase in these tissues in diabetics and may contribute to the development of diabetic complications. The etiology of the development of diabetic complications and the role of sorbitol in this process remain to be clarified. Nevertheless, the fact that inhibitors of ALR2 can prevent a number of diabetic complications in animal models of diabetes strongly supports the conclusion that the polyol pathway plays a role (15). Inhibition of glucose reduction through the use of inhibitors of ALR2 (usually called aldose reductase inhibitors) represents a possible method for prevention of diabetic complications in man. Numerous aldose reductase inhibitors are currently in clinical testing (15, 16). Most studies of potential aldose reductase inhibitors at the enzyme level have used non-human sources of ALR2 because of the instability of human ALR2 (16). It would be preferable to use human ALR2 for these studies because of marked species differences in the aldose reductase inhibitor-binding properties of ALR2.

In the present study, we report the development of a scheme for the rapid isolation of ALR1 and ALR2 from human placenta. We also report that there are two forms of ALR2 and that the ALR1/ALR2 ratio varies widely. The substrate specificities and drug-binding properties of ALR1 and ALR2 are compared.

EXPERIMENTAL PROCEDURES

Materials—Fresh human placenta, obtained from the University of New Mexico Hospital, were perfused with cold phosphate-buffered saline before being homogenized and stored at -70°C until use. Enzyme assays were performed at a pH of 7.0 and at 37°C. Each assay mixture contained 50 mM potassium phosphate buffer, 0.1 mM NADPH, and 100 nM ALR2. The redox state of the assay system was monitored with a spectrophotometer at 340 nm.

1. The abbreviations used are: ALR1, aldehyde reductase; ALR2, aldose reductase; DTT, dithiothreitol; HPLC, high performance liquid chromatography.
Enzyme Assays—Routine assays of reductase activities during purification of ALR1 and ALR2 were carried out in 1-ml volumes of 0.1 M sodium phosphate buffer, pH 7, containing 0.1 M NADPH and 10 mM n-l-glyceraldehyde. Reactions were monitored at 340 nm, 25°C, with a Beckman model X spectrophotometer. Kinetic studies of substrate specificities of the isolated reductases were carried out in the same buffer. The Hill numbers were obtained from nonlinear regression analysis of the initial rate data using the Enzfitter program (Elsevier-Biosoft).

Enzymochemistry—Rabbit antiserum against placenta ALR2 and against ALR1 that was purified from human liver (17). The antisera were titered against ALR1 and ALR2 from placenta in the same way as for the purified enzymes. This afforded a rapid and accurate method for determining the enzyme activities in placenta homogenates. ALR1 and ALR2 from two representative placenta samples are shown in Fig. 1. It is apparent that there are two peaks of reductase activity in placenta homogenates. The first peak is at pH 5.9, and the second at pH 5.5. Each peak of reductase activity was obtained in less than a 5-ml volume. Recoveries of activity were generally 75-80% total.

RESULTS

Purification of ALR1 and ALR2—The utilization of Red Sepharose for the extraction of all NADPH-dependent reductases that catalyze the reduction of glyceraldehyde provided a rapid first step in the purification of multiple reductases from a single sample of human placenta. Recoveries of activity were generally 100% and sometimes greater than 100%, which suggests that some placentas may have an inhibitory substance that is removed during this step of the purification scheme. The highest recovery of activity was 157%. Concentration of the eluent from Red Sepharose chromatography, 200 ml, to 5 ml by pressure filtration on an Amicon YM-10 membrane gave 95% recovery of activity. This is the only concentration step in the purification scheme. The rapid desalting of the concentrated sample by the addition of 2 x 2.5-ml fractions to a Pharmacia PD-10 desalting column gave 100% recovery in a total volume of 7 ml.

The second step in the purification scheme utilized chromatofocusing on Pharmacia PBE 94 resin. The entire 7-ml sample from the first step was added to the chromatofocusing column. Profiles from two representative samples are shown in Fig. 1. It is apparent that there are two peaks of reductase activity, and that the location of the peaks can vary widely. The first peak focused at pH 5.9 and the second at pH 5.5. Each peak of reductase activity was obtained in less than a 5-ml volume. Recoveries of activity were generally 75-80% total.
In the last step of the purification scheme, the two peaks of activity obtained from chromatofocusing were individually chromatographed in 0.5-ml volumes using a Bio-Gel HPHT hydroxylapatite HPLC column. A representative profile for the purification of the peak of activity which focused at pH 5.5 is shown in Fig. 2A. Chromatography of the peak of activity which focused at pH 5.9 is shown in Fig. 2B. A single peak was observed in each case. Inclusion of NADP in the elution buffer resulted in sharper peaks of activity. Recoveries of activity from the HPLC step of the purification scheme were 75–85%. In all of the purification steps, beginning with the elution of reductase activity from the Red Sepharose column, NADP was present. This markedly stabilized the enzymes. However, the purified enzymes were stable for at least 1 month at –20 °C in the absence of NADP. The enzymes are labile to repeated freezing and thawing.

The two reductases were homogeneous by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The reductase that focused at pH 5.5 migrated as a 38.9-kDa protein. The reductase that focused at pH 5.9 migrated as a 36.7-kDa protein. Only the 36.7-kDa reductase was activated (2-fold) by the presence of 0.4 M (NH₄)₂SO₄ in the assay buffer. Sulfate material from homogenization and centrifugation of human placenta was eluted with reductase activities as described above, providing a method for the rapid determination of the reductase profile of 100,000 x g supernatant. The enzymes are labile to repeated freezing and thawing.

Heterogeneous Expression of ALR1 and ALR2 in Human Placenta—Addition of samples of the 100,000 X g supernatant material from homogenization and centrifugation of human placenta directly onto the hydroxylapatite HPLC column and elution of reductase activities as described above provided a method for the rapid determination of the reductase profile of 100,000 X g supernatant. The reductase activity that was present in the elution buffer was used in the present of proteinase inhibitors and that the HPLC step could be completed within a few h from the time the placenta was obtained, it is likely that the profile of reductase activity is an accurate reflection of the in vivo distribution of ALR1 and ALR2 in human placenta.

The distribution of ALR1 and ALR2 in a random selection of 10 human placentas was determined using this rapid screening procedure. There is marked heterogeneity in the distribution of these enzymes. In some samples, ALR1 is predominant; in other samples ALR2 is the major form. Among the samples that were examined, the ALR1/ALR2 ratio ranged from 1:4 to 4:1. The starting specific activities of the total reductase activity in the 100,000 X g fraction also vary, ranging from 2 to 7 nmol/min/mg. Thus, we see that both the level of reductase activity and the ratio of ALR1 to ALR2 vary widely in human placenta.

Immunochemical Analysis of ALR1 and ALR2—Antisera were raised to placenta ALR2 and liver ALR1 in rabbits. Liver expresses mainly ALR2 (2). The two antisera were titered against placenta ALR1 and ALR2 by an enzyme-linked immunosorbent assay analysis. The results are shown in Fig. 3. Antiserum to liver ALR1 recognizes placenta ALR1 and liver ALR1 equally well but shows very weak cross-reactivity with ALR2. Likewise, antiserum to ALR2 is specific for ALR2, showing very little cross-reactivity. Therefore, ALR1 from liver and placenta appears to belong to an immunochemically distinct family from ALR2.

Substrate Specificity of ALR1—The substrate properties of ALR1 were evaluated with a range of substrates that included small hydrophilic aldehydes, such as glyceraldehyde, and larger hydrophilic aldehydes, such as p-nitrobenzaldehyde. The results are shown in Table I. ALR1 discriminates among aldehyde substrates mainly in the binding parameter K_m, which varies by more than 10^4. The k_cat values vary by only a factor of 5. The high discrimination in binding results in k_cat/K_m values that vary by 10^5 between good substrates, such as p-nitrobenzaldehyde, and poor substrates, such as glucose.

ALR1 shows substrate inhibition with most of its substrates. Fig. 4, which shows the pattern of substrate inhibition observed with glucuronic acid, is representative. As a consequence of substrate inhibition, the determination of specific activities under saturating conditions can give varying values, depending upon the concentration of the substrate. The k_cat values in Table I were determined by nonlinear regression analysis of the initial rate data that were obtained under nonsaturating conditions. The line that is shown in Fig. 4...
Table I
Substrate specificities of aldehyde reductase and aldose reductase from human placenta

<table>
<thead>
<tr>
<th>Enzyme and substrate</th>
<th>kcat</th>
<th>Km</th>
<th>kcat/Km</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALR1 Glyceraldehyde</td>
<td>560</td>
<td>3.4</td>
<td>1.7 x 10^6</td>
</tr>
<tr>
<td></td>
<td>565</td>
<td>0.069</td>
<td>9.5 x 10^6</td>
</tr>
<tr>
<td></td>
<td>246</td>
<td>2500</td>
<td>9.8 x 10^1</td>
</tr>
<tr>
<td></td>
<td>1950</td>
<td>3.6</td>
<td>3.5 x 10^9</td>
</tr>
<tr>
<td></td>
<td>1.95</td>
<td>0.004</td>
<td>0.004</td>
</tr>
</tbody>
</table>

ALR2 Glyceraldehyde  | 109  | 0.007 | 1.6 x 10^7 |
|                      | 38   | 0.002 | 4.9 x 10^2 |
|                      | 64   | 70    | 9.1 x 10^2 |
|                      | 81   | 2.8   | 2.9 x 10^4 |
|                      | NADPH |      | 0.002   |

FIG. 4. Kinetic profile of ALR1 with glucuronate as a representative substrate. Substrate inhibition is a common feature of the kinetics of ALR1. kcat and Km values were determined by nonlinear regression analysis of the experimental points up to 10 mM glucuronate.

(inset) was drawn by using the kcat and Km values determined by nonlinear regression analysis.

Substrate Specificity of ALR2—The substrate properties of ALR2 were evaluated with the same set of substrates as was used with ALR1. The results are shown in Table I. ALR2 discriminates among substrates almost entirely in the binding step since kcat values are essentially independent of substrate. With the same set of substrates, kcat and Km values are consistently lower for ALR2 than ALR1. The important substrate glucose is poor for ALR2 compared with other substrates. However, the Km for glucose (70 mM) suggests that ALR2 should be able to catalyze the reduction of glucose under physiological conditions, especially during hyperglycemia, when glucose concentrations can exceed 25 mM.

The characteristic pattern of substrate inhibition observed with ALR1 is not seen with ALR2. ALR2 shows variable patterns of response to high concentrations of substrate. For most substrates, ALR2 exhibits hyperbolic kinetics. A typical example is shown in Fig. 5 with p-nitrobenzaldehyde. In this example, the kcat value calculated by nonlinear regression analysis agrees with the value measured directly at high concentrations of substrate. With glyceraldehyde as substrate, however, ALR2 exhibits apparent substrate activation, as shown in Fig. 6A. At the lower end of the concentration range, the kinetic data reflect a Km = 7 μM. This unusual behavior was observed only with glyceraldehyde as substrate, as shown in Fig. 6B. Substrate activation was no longer observed. DTT-treated ALR2 showed hyperbolic kinetics with Km = 7 μM. The catalytic properties of ALR2 with other substrates were not affected by the DTT treatment. GSH could not replace DTT. It appears, therefore, that ALR2 is capable of existing in two forms that are related by disulfide bonds but that this does not alter the kinetic properties of ALR2 except with glyceraldehyde as substrate.

Inhibitor Binding to ALR1—Sorbinil (2,4-dihydro-6-fluorospiro[4H-1-benzopyran-4,4'-imidazolidine]2',5'-dione), tolrestat (N-[[6-methoxy-5-(trifluoromethyl)-1-naphthalenyl]thioxomethyl]-N-methylglycine), and statil (3-(4-fluorophenyl)methyl)-3,4-dihydro-4-oxo-1-phthalazinecarboxylic acid), three inhibitors of aldose reductase which are in clinical testing, were evaluated as inhibitors of ALR1. The results are listed in Table II. All three inhibitors were classical noncompetitive inhibitors of NADPH binding. The drug-binding site did not discriminate among the inhibitors. In addition, the inhibition constants were not very sensitive to changes in substrate. The largest variation in K values with different substrates was observed with sorbinil, in which K values varied 3-fold.

Inhibitor Binding to ALR2—The same three compounds were evaluated as inhibitors of ALR2; the results are shown...
The present study was designed to develop a scheme for the rapid isolation of ALR1 and ALR2 from human tissue which would provide forms of the enzymes whose properties were not altered during the purification process. Isolation of unaltered enzymes, especially ALR2, has been a problem. For example, both human ALR2 (16) and bovine ALR2 (22) have been shown to alter their kinetic or drug-binding properties during purification or during storage. There are major differences in the drug-binding properties of ALR2 from different species. It would be preferable to use human ALR2 for studies of potential aldose reductase inhibitors. In the past, evaluation of aldose reductase inhibitors has generally been carried out with rat lens ALR2 because of problems with the stability of human ALR2.

The scheme developed in the present study provided human placenta ALR1 and ALR2 rapidly and efficiently. The purification can be completed in 2 days, and the isolated enzymes are stable during storage. In addition, the kinetic and the drug-binding properties of ALR1 and ALR2 did not appear to have been altered during the purification. This conclusion is based upon the fact that the properties of ALR1 purified from different samples were the same. Likewise, the properties of ALR2 from multiple samples were the same. To test this conclusion further, the sensitivity of ALR2 to inhibition by sorbinil was measured both with the 100,000 × g supernatant fraction from human placenta and with purified ALR2. The Kᵢ values were almost identical. We conclude, therefore, that the isolation scheme described in this study provides human ALR1 and ALR2 whose properties have remained intact.

The variability in the ratio of ALR1 to ALR2 from different placenta as well as the differences in the reductase activities in different placenta indicate that there is considerable heterogeneity in the human population for expression of ALR1 and ALR2. Whether or not this variability extends to other tissues must still be determined. A number of studies have described the distribution of ALR1 and ALR2 in human tissues, based on immunohistochemical measurements (3, 29). However, these studies generally did not involve large numbers of samples; consequently, the question of heterogeneity must still be evaluated. In some cases, all of the data provide a consistent picture. For example, numerous studies indicate that human liver expresses mainly ALR1 and little or no ALR2 (23, 24). By contrast, the data for human erythrocytes are confusing, with several groups reporting that there is no ALR2 in the erythrocyte (3, 23) whereas others have described the isolation and properties of ALR2 from human erythrocytes (25). The possibility that these results reflect variability in the human population should be examined.

Evaluation of the variability in expression of ALR1 and ALR2 is potentially very important. Numerous studies support the idea that production of sorbitol is a critical step in the development of diabetic complications (26). If ALR2 but not ALR1 is essential for synthesis of sorbitol, then the tissue level of ALR2 may be the critical factor in determining whether a given diabetic will develop complications. Possibly, ALR2 profiles will be useful for predicting which diabetics are likely to develop complications and are therefore likely to benefit from aldose reductase inhibitor therapy.

Recently, structural data for human liver and placenta ALR1 and for human placenta ALR2 were reported, based upon cDNA sequences (27). It appears that liver ALR1 is identical to placenta ALR1 and that human DNA has a single ALR1 gene. The predicted amino acid sequence indicated a protein of M, 36,576. The quantitative enzyme-linked immunosorbent assay results in the present study (Fig. 3) are

![Diagram of Dixon plots of the inhibition of ALR2 by sorbinil.](http://www.jbc.org/) Deviations from classical noncompetitive inhibition are observed when sorbinil is the substrate. The extent of these deviations varies with different substrates and inhibitors, and the deviations are not altered by treatment of ALR2 with DTT.

**TABLE II**

<table>
<thead>
<tr>
<th>Enzyme and substrate</th>
<th>Sorbinil</th>
<th>Tolrestat</th>
<th>Statil</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALR1</td>
<td><img src="http://www.jbc.org/" alt="Values for sorbinil" /></td>
<td><img src="http://www.jbc.org/" alt="Values for tolrestat" /></td>
<td><img src="http://www.jbc.org/" alt="Values for statil" /></td>
</tr>
<tr>
<td>ALR2</td>
<td><img src="http://www.jbc.org/" alt="Values for sorbinil" /></td>
<td><img src="http://www.jbc.org/" alt="Values for tolrestat" /></td>
<td><img src="http://www.jbc.org/" alt="Values for statil" /></td>
</tr>
</tbody>
</table>

*Inhibition of ALR1 with glucose as substrate was not determined due to the poor substrate properties of glucose.*
consistent with the conclusion that liver and placenta express the same ALR1. The size of placenta ALR1 determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis is in reasonable agreement with the predicted size of ALR1.

Analysis of cDNA for placenta ALR2 indicated a protein, M, 35,858, which agrees with the results from the present study (M, 36,700). Southern analysis indicated that human ALR2, unlike ALR1, probably exists as a multigene family (27). Similar results have been reported from studies of rat DNA (28). We have observed a single form of ALR2 in placenta. However, a recent study of ALR2 from human muscle described a form of ALR2 that is related immunochemically to placenta ALR2 but shows markedly different kinetic and drug-binding properties compared with those described in the present study. In particular, glucose was a very poor substrate for muscle ALR2, \( K_m = 651 \text{ mM} \) (29). The possibility that muscle expresses a different form of ALR2 than placenta must be evaluated. A number of studies of ALR2 from human brain have described properties similar to those of placenta ALR2 (19, 30, 31). One of these studies, however, indicated that ALR2 in human brain is insensitive to sorbinil, in contradiction with the results (Table II) from the present study. Thus, the question of heterogeneity of human ALR2 needs to be evaluated both in terms of variability in tissues from different donors and in terms of the possible existence of isoenzyme forms with different kinetic properties, especially with glucose as substrate.

The nonlinear kinetics of ALR2 from various sources has been reported previously (32, 33). These studies were carried out using bovine lens ALR2 with glyceraldehyde as substrate. It has been suggested that the complex kinetics may be related to the autooxidation of glyceraldehyde leading to production of reduced oxygen species that may contribute to the oxidation of NADPH, thereby complicating the kinetics (33). In recent studies of bovine kidney ALR2, it has been shown that the biphasic kinetics result from the presence of two conformations of ALR2 with different kinetic properties (22). Other recent studies of bovine lens ALR2 suggested that enzyme oxidation resulted in the formation of a form of ALR2 with increased activity and decreased sensitivity to AR1 (34). In the present study, placenta ALR2 also exhibited nonhyperbolic kinetics with glyceraldehyde (Fig. 6A). This could be interpreted as evidence for the presence of two forms of ALR2. However, hyperbolic kinetics were observed with the other substrates that were used in this study. In addition, ALR2 that was isolated from different placentas gave the same pattern shown in Fig. 6A. There was no evidence that two forms were obtained in different ratios from different placentas. We conclude that a single form of human aldose reductase was isolated from placenta and that substrate activation by glyceraldehyde is an as yet unexplained kinetic characteristic of the enzyme.

Treatment of placenta ALR2 with DTT did not alter its kinetic properties with most substrates or alter its drug-binding properties. However, this treatment converted ALR2 into a form that showed hyperbolic kinetics with glyceraldehyde. The kinetic properties after reduction with DTT appeared to be the same as those observed at the lower glyceraldehyde concentrations in Fig. 6A. It appears that substrate activation of aldose reductase by glyceraldehyde is dependent upon the thiol-disulfide state of the enzyme.

It has been shown previously that the affinity of ALR2 for a particular aldose reductase inhibitor is substrate dependent (35). For example, bovine lens ALR2 binds sorbinil with a \( K_i \) of approximately 100 \( \mu \text{M} \) with \( p \)-nitrobenzaldehyde as substrate and 1 \( \mu \text{M} \) with glucose as substrate. Placenta ALR2 also shows some variation of \( K_i \) values with changes in the substrate that is used (Table II). However, the range of values is much smaller than was reported for ALR2 from bovine lens. For each inhibitor, the range of \( K_i \) values with changes in substrate was less than a factor of 4. It would appear, therefore, that there is less interaction between the substrate-binding site and the aldose reductase inhibitor-binding site on placenta ALR2 compared with ALR2 from other sources.

In summary, we have described a scheme for the rapid and efficient isolation of ALR1 and ALR2 from human placenta and have described some of the kinetic and drug-binding properties of these enzymes. These enzymes appear to have retained their in vivo properties and should therefore be useful for studies of aldose reductase inhibitors. We have pointed to a number of important questions related to heterogeneous expression of ALR1 and ALR2 in the human population and the possible importance of this variability in the development of diabetic complications. The purification scheme described for placenta ALR1 and ALR2, if applicable to other human tissue, should provide the methodology for answering some of these questions.

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Aldehyde and aldose reductases from human placenta. Heterogeneous expression of multiple enzyme forms.

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