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In vitro expression of rat lens aldose reductase in *Escherichia coli*

(diabetic complications/polyol pathway/recombinant enzyme activity)

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ABSTRACT **Aldose reductase (alditol:NADP$^+$ oxidoreductase, EC 1.1.1.21), an enzyme that converts glucose to sorbitol, the first step of the polyol pathway, has been implicated in secondary complications of diabetes, such as cataracts, retinopathy, neuropathy, and nephropathy. Aldose reductase inhibitors have been observed to prevent or delay the onset of these complications; however, more potent and specific inhibitors are needed. Development of new inhibitors necessitates a better understanding of the molecular structure of this protein. To elucidate the structure–function relationships of aldose reductase and to develop methods of regulating this enzyme, large and homogeneous quantities of rat lens aldose reductase have been expressed in bacterial cells. A construction of the complete coding sequence and 3′ untranslated region for rat lens aldose reductase was assembled in the expression vector pKK233-2 (Pharmacia). This construction expresses an active enzyme that has been purified and demonstrates kinetic, immunological, and inhibitory properties similar to rat lens aldose reductase.**

Aldose reductase (alditol:NADP$^+$ oxidoreductase, EC 1.1.1.21) is the first enzyme in the polyol pathway. It reduces glucose to sorbitol, which can then be converted to fructose by sorbitol dehydrogenase (EC 1.1.1.14). The accumulation of sorbitol has been linked to diabetic complications affecting the nerves, lens, retina, and kidneys (1–4). In hyperglycemia, the accumulation of intracellular sorbitol causes an influx of water, which results in swelling and eventual loss of cellular integrity (3, 5). The regulation and expression of aldose reductase have been examined by a number of investigators in the kidney (6–10), in the lens (11–13), and in the placenta (14, 15).

The involvement of aldose reductase in the secondary complications of diabetes has led to the development of a number of aldose reductase inhibitors. Inhibition of aldose reductase has been shown to be effective in reducing diabetic complications (16–21). However, better knowledge of the detailed structure of aldose reductase may aid in the development of more specific, potent inhibitors.

Studies on the regulatory properties, potential inhibitor sites, and overall structure of aldose reductase requires large amounts of purified enzyme. Here we report the construction of the complete coding sequence for rat lens aldose reductase in a bacterial expression vector and the subsequent production, purification, and characterization of this recombinant enzyme.

MATERIALS AND METHODS

Plasmid Construction. A full-length DNA sequence corresponding to the coding region and the 3′ untranslated sequences of the rat lens aldose reductase cDNA was constructed in the expression vector pKK233-2 (Pharmacia) (Fig. 1). The cDNA clone 4B (22) was digested with *Ban* I and the 990-base-pair (bp) fragment was purified from a 4% polyacrylamide gel. An oligonucleotide was synthesized (Applied Biosystems DNA synthesizer, model 380B) and purified (from a 4% polyacrylamide gel) that corresponded to the 56 bp of the 5′ coding sequence (from the ATG initiation codon to the *Ban* I site at the 3′ end) plus 10 bp that included a *Bam*HI site and a *Hin*dIII overhang site on the 5′ end of the oligonucleotide. The oligonucleotide was ligated to the *Ban* I fragment of 4B and gel purified from a 7% agarose gel using NA45 nitrocellulose according to manufacturer's instruction (Schleicher & Schuell). This new *Hin*dIII fragment was ligated into pBSII KS− (Stratagene). In this construction there was anomalous cloning at the 3′ end of the construct. The oligonucleotide did not ligate to the 3′ end of the 4B fragment, and the *Ban* I site from the 3′ end of 4B and the *Hin*dIII site from the vector ligated together destroying both recognition sequences (Fig. 1, Ø). One positive clone (pBSH8) was digested with *Nco* I and *Pst* I and the 303-bp fragment was purified (NA45; Schleicher & Schuell). This *Nco* I–*Pst* I fragment was then ligated into the expression vector pKK233-2 (Pharmacia) (construct pKKNP1). The cDNA clone 10Q (23) was digested with *Pst* I and the 1044-bp fragment was purified (NA45; Schleicher & Schuell). This *Pst* I fragment corresponded to the remaining 649 bp of the coding sequence, the 3′ untranslated region, and the putative poly(A) sequence. The *Pst* I fragment was ligated into the *Pst* I restriction enzyme site in the previous construct (pKKNP1). This *Pst* I fragment was incorporated in both orientations. Two constructs had ligated the *Pst* I fragment in the forward orientation (pKKARX28, pKKARX33) and two in the reverse orientation (pKKARX22, pKKARX36) with respect to the expression vector's promoter, trc (a hybrid trp-lac promoter, ref. 24). All cloning was performed in *Escherichia coli* DH5α (Bethesda Research Laboratory). Construct pKKARX33 was sequenced to ensure no cloning artifacts were produced during the multiple cloning steps (Sequenase kit; United States Biochemical). Construct pKKARX28 was also sequenced through the ligation sites.

Antibody Reactions. The *E. coli* strains harboring the aldose reductase constructs were grown in 10 ml of Luria broth plus 50 μg of ampicillin per ml overnight. The cells were centrifuged (Sorvall RC6000; 2000 × *g*) and washed twice in 20 mM phosphate buffer containing 7 mM 2-mercaptoethanol and 0.5 mM EDTA (pH 7.5). The cell pellet was resuspended in 0.5 ml of the same buffer plus 1 mM phenylmethylsulfonyl fluoride (PMSF; Sigma) and sonicated (five bursts, 2- to 3-sec pulses at 50% power) to break the bacterial cell walls. The Bradford protein assay (Bio-Rad) was performed to determine protein concentration using bovine serum albumin as a standard. Two micrograms of total protein was separated on the Phastgel system (Pharmacia) according to manufacturer's instructions. The protein gels were either stained with Coomassie blue or transferred to nitrocellulose for immunoblot analysis.

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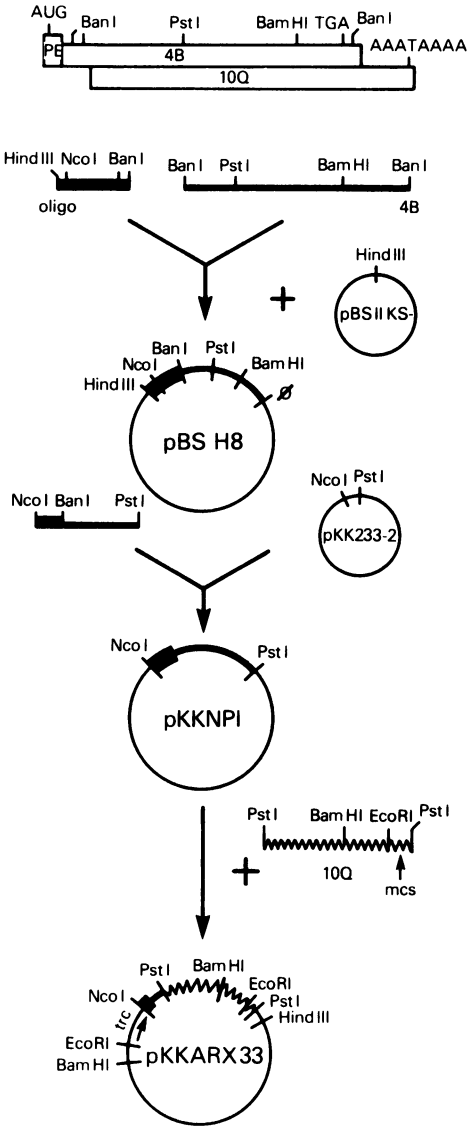

FIG. 1. Schematic diagram of the construction of pKKARX33. Details of the construction are found in the text. PE, primer extension; mcs, multiple cloning site; AUG, start codon; TGA, stop codon; AATAAA, poly(A) sequence; trc, expression vector promoter; Ø, loss of restriction enzyme site. Restriction enzyme sites labeled: *Bam*HI, *Ban* I, *Eco*RI, *Hin*dIII, *Nco* I, and *Pst* I.

The nitrocellulose filters were incubated with a rat lens aldose reductase antibody (1:250 dilution) (25) overnight at 4°C after 30 min in a 0.2% milk blocking solution. To visualize the antigen–antibody complex the Vectastain ABC kit was used according to manufacturer's instructions (Vector Laboratories). Recombinant aldose reductase protein was compared to purified rat kidney aldose reductase and whole rat lens homogenate.

Protein Purification. *E. coli* DH5α harboring construct pKKARX33 was grown in 500 ml of Luria broth plus 50 μg of ampicillin per ml overnight. The cells were centrifuged (GS3; 8000 × *g*) and washed twice in 20 mM phosphate buffer containing 7 mM 2-mercaptoethanol and 0.5 mM EDTA (pH 7.5). The cell pellet was resuspended in 5 ml of the same buffer plus 1 mM PMSF and split into five tubes for sonication (10 bursts for 3–5 sec at 50% power). The sonicated samples were pooled and protein concentration was determined by the Bradford protein assay (Bio-Rad). The sample centrifuged at 10,000 × *g* for 15 min and the recombinant protein was then purified from the supernatant as described (26). Briefly, the supernatant was applied to a Sephadex G-75 column and eluted with the same phosphate buffer as above. Fractions collected (about 10 ml) were assayed for NADPH reducing activity with DL-glyceraldehyde as substrate. Those fractions containing activity were applied to a Matrex gel orange A column (Amicon) equilibrated with the same phosphate buffer. After washing with 300 ml of the phosphate buffer followed by 200 ml of 10 mM imidazole buffer (pH 7.6) containing 7 mM 2-mercaptoethanol, the recombinant protein was eluted with 100 ml of the imidazole buffer containing 0.1 mM NADPH and collected in 150 drop fractions. Fractions containing enzyme activity were applied to a Mono P column (Pharmacia) and eluted with Polybuffer 74 (Pharmacia) containing 7 mM 2-mercaptoethanol. The purification process was also performed using the DH5α cells as a control.

Aldose Reductase Activity and Inhibition. Aldose reductase activity was photometrically determined by monitoring the decrease in absorbance of NADPH at 340 nm using 10 mM DL-glyceraldehyde, 10 mM D-xylose, 10 mM D-glucuronate, 10 mM D-galactose, or 10 mM D-glucose as substrates. One unit of activity was defined as the activity consuming 1 μmol of NADPH per min at 22°C.

Inhibition of aldose reductase by Sorbinil (Pfizer), AL 1576 (Alcon), Tolrestat (Wyeth-Ayerst), and Ponalrestat (ICI) was determined with DL-glyceraldehyde as substrate as described (27).

Recombinant aldose reductase activity and inhibition were compared to activity and inhibition of purified rat lens aldose reductase.

RESULTS

Two constructions were made that correspond to the full-length DNA coding sequence and 3′ untranslated region of the rat lens aldose reductase (pKKARX28 and pKKARX33) (outlined in Fig. 1 and *Materials and Methods*). In addition, two constructs were made that contained a large portion of the cDNA in reverse orientation with respect to the trc promoter (pKKARX22 and pKKARX36) and one construct was made that contained only the first 303 bp of the coding sequence (pKKNP1). These constructions were introduced into *E. coli* DH5α where the trc promoter is constitutively expressed. The constitutive expression of aldose reductase did not appear to affect bacterial cell growth. The immuno-

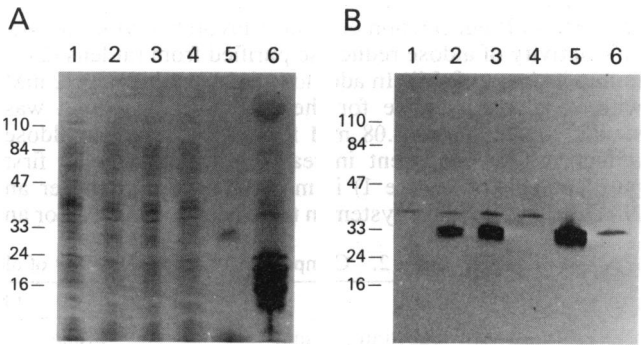

FIG. 2. SDS/PAGE and immunoblot of the rat lens aldose reductase constructs. (*A*) Two micrograms of total protein was separated on an SDS/12.5% polyacrylamide gel. (*B*) The proteins were transferred to nitrocellulose and probed with antiserum to rat lens aldose reductase. Lanes: 1, DH5α cells with no plasmid; 2 and 3, constructs pKKARX28 and pKKARX33, respectively, with the insert in the forward orientation relative to the promoter; 4, construct pKKARX36 with the insert in the reverse orientation relative to the promoter; 5, purified rat kidney aldose reductase (0.25 μg of purified protein); 6, whole rat lens homogenate (8 μg of total protein). Apparent molecular weights are indicated as $M_r \times 10^{-3}$.

Table 1. Purification of aldose reductase from pKKARX33

Fraction	Total protein, mg	Specific activity, milliunits/mg	Total activity, units	Purification, fold	Yield, %
Homogenate	125.2	42.1	5,284	1	100
G-75	26.0	427.6	11,116	10.1	210.4
Orange A	2.04	2910.8	5,939	69.0	112.4
Mono P	1.34	3008.9	4,032	71.5	76.3

Recombinant rat lens aldose reductase was purified from DH5α cells containing pKKARX33 in a stepwise fashion. The bacterial cells were sonicated (homogenate) and purified by gel filtration on a Sephadex G-75 column (size separation), an Amicon Matrex gel orange A column (NADPH affinity purification), and a Mono P column (chromatofocusing).

blot analysis of the various constructs showed that only pKKARX33 and pKKARX28 had an additional protein that reacted with the rat lens aldose reductase antibody (Fig. 2). This protein had an approximate M_r of 35,000 (Fig. 2*B*, lanes 2 and 3), which is similar to purified rat kidney and whole rat lens aldose reductase (Fig. 2*B*, lanes 5 and 6). Constructs pKKNP1 (unpublished, S.E.O.), pKKARX22 (unpublished, S.E.O.), and pKKARX36 (Fig. 2*B*, lane 4) as well as DH5α alone (Fig. 2*B*, lane 1) did not demonstrate this additional protein. The bacterial cells did have a protein that reacted with the antibody with a M_r of ≈41,000. This higher molecular weight protein was seen in all of the constructs as well as the original bacterial strain. This high molecular weight protein had some aldose reductase-like activity but was easily separated from the recombinant protein by gel filtration on the Sephadex G-75 column (unpublished, S.S.).

The trc promoter in these constructs can be repressed in *E. coli* JM105 cells and induced by 2.5 mM isopropyl β-D-thiogalactoside (IPTG). Time course experiments were performed in IPTG-induced JM105 with similar SDS/PAGE and immunoblot results as seen in the *E. coli* DH5α cells (immunoreactive recombinant protein was detected by 2 hr following induction; unpublished, S.E.O.). Since the constitutive expression of the recombinant protein did not harm the cells, the DH5α bacterial strain was used for all of the experiments described here.

The purification steps for the pKKARX33 recombinant protein are summarized in Table 1. The recombinant protein roughly accounts for 4% (based on percent yield and total protein) of the total protein in these cells. The specific activity of 3009 milliunits/mg for the recombinant protein after Mono P purification compares favorably with the specific activity of aldose reductase purified from rat lens (2433 milliunits/mg; ref. 26). In addition, the K_m value of 0.12 mM with DL-glyceraldehyde for the recombinant protein was similar to the K_m of 0.08 mM for purified rat lens aldose reductase. The apparent increase in yield after the first purification step (Table 1) is most likely due to either an NADPH regeneration system in the whole homogenate or an endogenous inhibition of the enzyme in the sonicated cells, either of which is removed following gel filtration. Purification of the recombinant protein on Mono P produced only one peak containing enzyme activity (Fig. 3), in contrast to the one major peak plus several smaller peaks observed with rat lens aldose reductase (26). The purified protein appeared as a single band on SDS/PAGE immediately upon purification. However, upon storage of the protein a doublet was observed. The second band was observed with the recombinant protein as well as the purified aldose reductase from kidney. The recombinant purified protein reacted with antibodies raised against rat lens aldose reductase (Fig. 3 *Inset*, lanes 1 and 4).

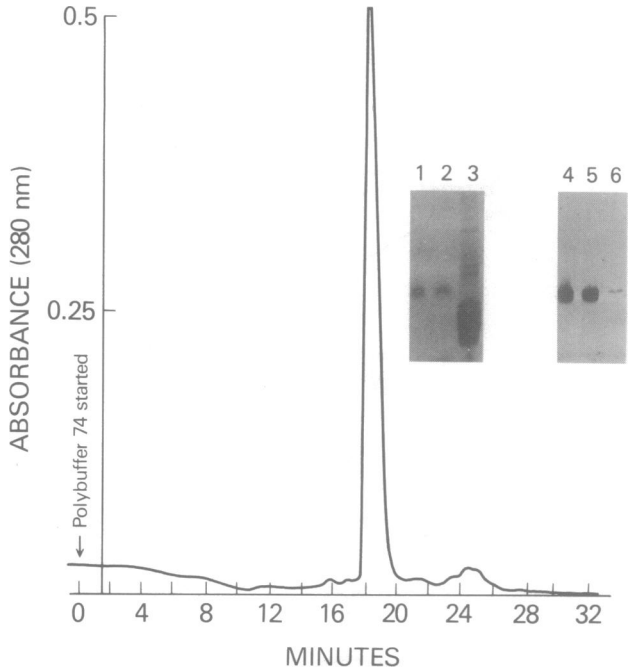

FIG. 3. Profile of the recombinant protein on a Mono P column. The recombinant protein eluted from the Matrex gel orange A column with NADPH was further purified on a Mono P column. One peak appears at 18–19 min after Polybuffer 74 is started. The flow rate is 1 ml/min. (*Inset*) The purity of the protein was examined by SDS/PAGE (lanes 1–3) and immunoblotting (lanes 4–6) with the rat lens aldose reductase antibody. Lanes: 1 and 4, 0.25 μg of purified recombinant aldose reductase from construct pKKARX33; 2 and 5, 0.25 μg of purified rat kidney aldose reductase; 3 and 6, 8 μg of total rat lens homogenate. Apparent molecular weights are indicated as $M_r \times 10^{-3}$.

The relative activities of the pKKARX33 crude cell homogenate and purified recombinant protein were tested with various substrates and compared to that of purified rat lens aldose reductase (Table 2), with the relative activity of DL-glyceraldehyde at 100%. The activity profiles of pKKARX-

Table 2. Comparison of relative activity of aldose reductase with different substrates

Substrate, 10 mM	pKKARX33, % Crude	pKKARX33, % Pure	RLAR, %
DL-Glyceraldehyde	100	100	100
D-Glucuronate	21.7 ± 3.2 (4)	21.3 ± 1.7 (5)	24.5 ± 2.9 (3)
D-Xylose	26.4 ± 0.8 (4)	23.0 ± 4.3 (5)	25.7 ± 1.1 (4)
D-Galactose	—	4.7 ± 0.8 (3)	5.5 ± 0.5 (4)
D-Glucose	—	1.7 ± 0.4 (3)	1.8 ± 0.5 (3)

The relative specificities of various substrates for aldose reductase were compared for the recombinant aldose reductase (pKKARX33) and purified rat lens aldose reductase (RLAR). The specific activity of DL-glyceraldehyde was set at 100%. Values are given as mean ± SD, with the number of samples in parentheses. The total cell homogenate (crude) and the purified recombinant aldose reductase from the Mono P column (pure) were examined.

Table 3. Inhibition of aldose reductase using 10 mM DL-glyceraldehyde as substrate

Compound	IC_{50}, μM	
	pKKARX33	RLAR
Sorbinil	0.165 ± 0.005 (3)	0.122 ± 0.004 (3)
AL 1576	0.016 ± 0.002 (4)	0.015 ± 0.008 (4)
Tolrestat	0.018 ± 0.002 (3)	0.023 ± 0.008 (4)
Ponalrestat (statil)	0.017 ± 0.002 (3)	0.010 ± 0.005 (3)

The IC_{50} values were determined for several aldose reductase inhibitors on the purified recombinant aldose reductase (pKKARX33) and on purified rat lens aldose reductase (RLAR). Values are given as mean ± SD, with the number of samples in parentheses.

33 protein and rat lens aldose reductase are nearly identical. The activity of the recombinant protein was also stimulated by the presence of 0.1 M sulfate ions [Li_2SO_4, Na_2SO_4, and $(NH_4)_2SO_4$] and decreased by 0.2 M chloride ions (NaCl). This is consistent with aldose reductase activity (28).

The recombinant enzyme was also inhibited by a series of aldose reductase inhibitors (Table 3). As noted by their IC_{50} values, these inhibitors produced similar inhibition in the recombinant protein and rat lens aldose reductase.

DISCUSSION

Mounting experimental evidence links the aldose reductase-initiated accumulation of polyol with the onset of diabetic complications (3). Furthermore, these studies suggest that aldose reductase inhibitors represent a pharmacologically-derived approach for the treatment of diabetic complications that is independent of the control of blood sugar levels. Preliminary clinical trials, however, suggest that more potent and selective aldose reductase inhibitors are required. To facilitate the development of additional inhibitors, knowledge of the inhibitor binding site(s) and the mechanism of action of these inhibitors is required. Such studies utilize large amounts of purified enzyme. Presently, significant amounts of animal tissue must be utilized in order to obtain the large amounts of purified enzyme necessary. However, the development of recombinant DNA technology provides an alternative to the use of large quantities of animal tissue.

The construction of a rat lens aldose reductase DNA coding sequence in an expression vector has yielded an abundant, enzymatically active enzyme in bacterial cells that is immunologically detectable by rat lens aldose reductase antibodies. This recombinant enzyme was purified to apparent homogeneity, demonstrated relative substrate specificities, and was inhibited by aldose reductase inhibitors similar to that of rat lens aldose reductase. The recombinant enzyme has a molecular weight similar to rat lens and rat kidney aldose reductase on SDS/PAGE and immunoblotting. Together, these data strongly suggest that the recombinant enzyme produced in these DH5α cells is rat lens aldose reductase.

The bacterial cells producing recombinant aldose reductase represent an approach for obtaining large quantities of enzyme that can be purified to apparent homogeneity. In these cells, aldose reductase constitutes ≈4% of the total proteins. The availability of this enzyme in abundant quantities by recombinant methods will facilitate studies on the characterization of the key elements of the substrate, NADPH, and inhibitor binding sites through the use of site-directed mutagenesis studies. Moreover, the recombinant enzyme represents an aldose reductase protein derived from a single coding sequence. This recombinant enzyme appeared as a single form on chromatofocusing as compared to several forms of the protein observed for rat aldose reductase isolated from tissue (26). This greater homogeneity should facilitate crystallization.

Many experimental studies on aldose reductase have utilized diabetic and galactosemic rats. In addition, aldose reductase extracted from rat lens has been extensively utilized for inhibitor structure–activity relationships and affinity labeling studies. Thus, the larger supplies of this specific enzyme, now available from our recombinant rat lens aldose reductase expression system, will benefit these studies. Overall, the expression of aldose reductase *in vitro* will facilitate the characterization of this enzyme in its normal physiological role and its pathological involvement in diabetes and galactosemia.

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