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SITE-DIRECTED MUTAGENESIS OF HIS-42, HIS-188 AND LYS-263 OF HUMAN ALDOSE REDUCTASE

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SUMMARY : The role of His^{42}, His^{188}, and Lys^{263} residues in the catalytic action of human aldose reductase was investigated in association with various inhibitors of this enzyme by site-directed mutagenesis. While mutations at His^{42}->Gln, His^{42}->Tyr, His^{188}->Gln, and His^{188}->Tyr brought small change in the kinetic parameters, Lys^{263}->Glu mutation markedly increased the K_m value for the substrate DL-glyceraldehyde by a factor of 60. Lys^{263}->Met substitution resulted in approximately 14 fold elevation of K_m for the substrate. By contrast, mutation of Lys^{263}->Arg significantly decreased the K_m for the substrate with concomitant reduction in k_{cat}. Moderate increase in K_m values for the cofactor NADPH was demonstrated for mutated enzymes. These results are indicative of the possible role of Lys^{263} in the substrate binding through electrostatic interaction. The inhibitor constants (K_i) for structurally diverse aldose reductase inhibitors against mutated enzymes demonstrated different degree of alteration, indicating binding sites of aldose reductase inhibitors on the enzyme molecule vary from one another, and some of the sites are more closely correlated with the physicochemical property of Lys^{263}.

Aldose reductase (EC 1.1.1.21) is a member of the monomeric NADPH dependent aldo-keto reductases which catalyze the reduction of various aldehydes, including the aldehyde form of glucose to the corresponding sugar alcohol, sorbitol. Since sorbitol accumulation in cells undergoing insulin-independent uptake of glucose has been demonstrated to play a key role in the pathogenesis of diabetic complications (1-5), the inhibitors for this enzyme have

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the potential to serve as a new means to prevent and treat these complications. A variety of aldose reductase inhibitors of diverse chemical structure has thus been developed, and some of them are already approved for the treatment of diabetic patients.

Previous studies on the primary structure of aldose reductase revealed considerable sequence similarity to other aldo-keto reductase family which shares overlapping substrate specificities and uses NADPH as a cofactor (6-8). The areas of sequence identity may accordingly indicate functional domains involved in the enzyme reaction common to these aldo-keto reductases. His^{42} and His^{188} are both located in highly conserved regions of aldo-keto reductase family and possess side chains which would act as proton carrier in the catalytic action of enzyme. On the other hand, an essential role for Lys^{263} in the active site of aldose reductase was suggested in the previous studies using pyridoxal reagents as affinity ligands (9).

To verify the role of these amino acid residues in the catalytic action and in the association with inhibitors for aldose reductase, we have introduced site-specific replacement of His^{42}, His^{188}, and Lys^{263} with amino acids differing in charge and polarity. The mutated human enzymes were expressed in a baculovirus-insect cell system, which has been verified to generate structurally and functionally identical enzyme form to native human aldose reductase (10).

MATERIALS AND METHODS

Oligonucleotide-directed Mutagenesis

The cDNA insert of human aldose reductase cDNA clone L-1 (11) was subcloned into the *Eco*RI site of M13mp8 and used as a template for the site-directed mutagenesis. Mutagenic oligonucleotides (Table I), made on an Applied Biosystems Model 391 DNA synthesizer and purified with OPC cartridges, were 5'-end phosphorylated and annealed to the single-stranded template. The reaction of mutagenesis was carried out using an oligonucleotide-directed *in vitro* mutagenesis system (Version 2; Amersham Corp.) under conditions described by the supplier. Mutated cDNA in M13 was verified by dideoxynucleotide sequencing using α-^{35}S-dATP (12), and replicative form of M13 was prepared for the isolation of mutated cDNA fragment.

Expression of Mutated Aldose Reductase in Baculovirus System

Following blunt end treatment of the primary *Eco*RI sites in baculovirus transfer vector pAcYM1 (11,13), a new *Eco*RI site was created in the vector downstream of its polyhedrin promoter. The mutated cDNA fragment isolated from M13 was introduced into this site, and the vector plasmid anchoring mutated cDNA in a 5'- to 3'-orientation against polyhedrin promoter was selected by the appearance of DNA products following PCR using one primer derived from pAcYM1 sequence and a second primer derived from the mutated cDNA. The integrity of the entire coding region of the mutated cDNA construct was further confirmed by direct nucleotide sequencing analysis using oligonucleotide primers and α-^{35}S-dATP. The recombinant baculovirus containing the mutated aldose reductase cDNA in the original viral genome was prepared as previously described (13). Purified recombinant virus stock of high titration ($>10^7$ plaque-forming unit/ml) was used to infect SF9 cells for the production of mutated human aldose reductase (10,11).

Purification of Mutated Aldose Reductase

Culture medium of SF9 cells containing recombinant enzyme protein was harvested at 3 days after infection. Following the addition of dithiothreitol (DTT) to the medium to give a final concentration of 5 mM, the medium was concentrated using Centriprep-10 (Amicon) and desalted with a Sephadex G-25 (Pharmacia LKB) column equilibrated with 20 mM sodium phosphate buffer (pH 7.4) and 2 mM DTT. Desalted samples were subsequently applied to hydroxylapatite high pressure liquid chromatography (HPLC) column (Tohnen, TAPS0508-05) equilibrated with the same buffer. The enzyme fraction eluted with a linear gradient from 20 mM to 500 mM sodium phosphate containing 2 mM DTT was collected from the column and subjected to SDS-polyacrylamide gel electrophoresis. Homogeneity of the enzyme preparation was verified by silver staining of the gel. All of the enzyme assays with purified enzyme were completed within 5 days after purification.

Kinetic Studies of Mutated Aldose Reductase

Standard reaction mixture for enzyme assay contained 0.1 M sodium phosphate buffer (pH 6.2), 150 μM NADPH, 10 mM DL-glyceraldehyde, and enzyme preparation in a total volume of 0.2 ml. The reaction was started by the addition of enzyme and activity was determined spectrophotometrically by measuring NADPH oxidation from the decrease in absorbance at 340 nm. All assays were performed at 25 °C using a JASCO Ubest-50 spectrophotometer (Japan Spectroscopic Co.Ltd.). To correct nonspecific oxidation of NADPH, the appropriate blank was subtracted from each assay. One unit of enzyme activity is defined as the amount of enzyme catalyzing the oxidation of 1 μmol of NADPH/min under the assay conditions. For the determination of kinetic parameters, the initial velocity was measured at 8 to 11 different concentrations of DL-glyceraldehyde or NADPH, and the mean value of 2 to 4 determinations of each concentration was used for the computer analysis fitting the data by the least-squares method. Aldose reductase inhibitors sorbinil and tolrestat were obtained from Dr. P. Kador (National Eye Institute, Bethesda), AL1576 from Dr. B. York (Alcon Laboratories, Inc., Fort Worth), and statil from Dr. T.G. Flynn (Queen's University, Kingston). Inhibitor constants were determined by Dixon plots at saturating concentration of NADPH (150 μM) and at variable concentrations (5-1000 μM) of the DL-glyceraldehyde. Protein concentration was measured by the method of Bradford (14).

RESULTS AND DISCUSSION

Preparation of Mutated Aldose Reductase

A single nucleotide replacement was introduced to convert His^{42} and His^{188} to Gln or Tyr, and Lys^{263} to Arg, Glu, or Met by oligonucleotide-directed mutagenesis (Table I). Recombinant aldose reductase containing each amino acid substitution was produced in a baculovirus-insect cell system and purified by HPLC using hydroxylapatite column. Hydroxylapatite was chosen for affinity resin instead of Matrex gel orange A used previously (10), because the affinity binding site for the dye-ligand on mutated enzyme is possibly disintegrated. Silver staining after SDS-polyacrylamide gel electrophoresis of the HPLC-eluate demonstrated a single band, indicating the homogeneity of the enzyme preparation purified by this method.

Kinetic Analyses of Mutated Aldose Reductase

The kinetic constants of mutated enzyme determined for a cofactor NADPH and the representative substrate DL-glyceraldehyde are outlined in Table II and III. Since the physiological substrate D-glucose has low affinity to the enzyme,

TABLE I

Oligonucleotides used for mutagenesis

	Gly^{39}	Tyr^{40}	Arg^{41}	His^{42}	Ile^{43}	Asp^{44}	Cys^{45}
Wild-type	5'-GGG	TAC	CGC	CAC	ATC	GAC	TGT -3'
His^{42}->Gln		TAC	CGC	CAG	ATC	GAC	TGT
His^{42}->Tyr	GG	TAC	CGC	TAC	ATC	GAC	T
	Ile^{185}	Glu^{186}	Cys^{187}	His^{188}	Pro^{189}	Tyr^{190}	Leu^{191}
Wild-type	5'-ATT	GAG	TGC	CAC	CCA	TAT	CTC -3'
His^{188}->Gln		GAG	TGC	CAG	CCA	TAT	CTC
His^{188}->Tyr	TT	GAG	TGC	TAC	CCA	TAT	C
	Val^{260}	Ile^{261}	Pro^{262}	Lys^{263}	Ser^{264}	Val^{265}	Thr^{266}
Wild-type	5'-GTG	ATC	CCC	AAG	TCT	GTG	ACA -3'
Lys^{263}->Met	G	ATC	CCC	ATG	TCT	GTG	A
Lys^{263}->Glu	G	ATC	CCC	GAG	TCT	GTG	A
Lys^{263}->Arg	G	ATC	CCC	AGG	TCT	GTG	A

DL-glyceraldehyde was used for the present kinetic analyses. Most of the mutated enzymes showed moderate alteration in the K_m values for the cofactor NADPH, Lys^{263}->Met substitution being most affected. While His^{42} and His^{188} mutation had small effect on the K_m values for the substrate glyceraldehyde, striking degree of alteration in the K_m of Lys^{263} mutation was demonstrated. Lys^{263}->Met mutation elicited approximately 14 fold elevation, whereas Lys^{263} ->Glu substitution induced as much as 60 fold increase in the K_m value for the substrate. This resulted in very low catalytic efficiency (k_{cat}/K_m) for the Glu mutant. By contrast, Lys^{263}->Arg substitution markedly diminished the K_m value for the substrate, which was almost 20 times less than that of the wild-type enzyme. Although k_{cat} of Arg mutant was concurrently reduced, the overall kinetic efficiency (k_{cat}/K_m) of Arg mutant was significantly augmented comparing with the wild-type form.

While replacement of His residues with amino acids differing in charge property had minute effect on the catalytic action of the enzyme, results on enzyme kinetics of Lys^{263} replacement with three amino acids bearing different

TABLE II

Effect of His^{42} or His^{188} mutation on kinetic parameters of aldose reductase

	DL-glyceraldehyde			NADPH
	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}M^{-1}$)	K_m (μM)
Wild-type	20.7	0.83	39900	4.74
His^{42}->Gln	43.8	0.52	11800	16.0
His^{42}->Tyr	45.9	1.07	23300	4.44
His^{188}->Gln	21.4	1.11	51900	9.54
His^{188}->Tyr	69.3	1.34	19300	7.54

TABLE III

Effect of Lys^{263} mutation on kinetic parameters of aldose reductase

	DL-glyceraldehyde			NADPH
	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}M^{-1}$)	K_m (μM)
Wild-type	15.5	0.72	46500	4.25
Lys^{263}->Met	211	0.69	3270	35.3
Lys^{263}->Glu	931	0.50	537	15.6
Lys^{263}->Arg	0.83	0.19	229000	21.1

physico-chemical properties indicated the essential role of this residue in enzyme reaction. In the previous work Morjana et al. (9) concluded this Lys residue located in the coenzyme binding site based on their findings on purified human muscle aldose reductase labeled with pyridoxal reagents. The present study, however, demonstrated lesser degree of alteration in K_m values for the cofactor NADPH against all of the Lys^{263} replaced enzymes. Instead, greater alteration was observed in the kinetic constants for the substrate DL-glyceraldehyde.

Met is one of the nonpolar amino acids which resembles Lys in general size and shape. Lys, Glu, and Arg are, on the other hand, amino acids with dissociable protons, which can directly affect the enzyme reaction. While Glu is an acidic amino acid, Lys and Arg are both basic amino acids, the latter being more basic than the former. Considering such difference in charge property among the replaced amino acids, one plausible interpretation of our data is that Lys^{263} is closely involved in substrate binding to the enzyme through electrostatic interaction.

Since alteration of aldose reductase activity induced by the modification of Lys^{263} with pyridoxal reagents was abolished in the presence of cofactor NADPH or its analog, the involvement of this reactive Lys in the cofactor binding was suggested in the previous study. Their work utilized pyridoxal 5'-phosphate and its derivative as affinity ligands which resulted in the formation of Schiff base complex between the aldehyde group of the pyridoxyl moiety and the ϵ-amino group of Lys^{263} (9). Thus, it was the aldehyde group of the affinity ligands which directly reacted with Lys^{263}. In this regard, our findings suggestive of substrate (aldehyde) binding to this Lys residue are in line with the previous study carried out by affinity labeling. Since the substrate binding site should reside in close proximity to the catalytic site where the cofactor interacts, the protective effect of NADPH against alteration in enzyme activity shown in their study may be attributable to an ordered sequential mechanism for this enzyme reaction. Lack of significant effect on the K_m values for NADPH revealed in Lys^{263} mutants clearly indicates diminutive involvement of this Lys residue in the cofactor binding.

TABLE IV

Effect of His^{42} or His^{188} mutation on inhibitor constants for aldose reductase

	K_i (μM)			
	Sorbinil	AL1576	Tolrestat	Statil
Wild-type	6.3	0.24	0.037	0.14
His^{42}->Gln	17.0	0.28	0.033	0.32
His^{42}->Tyr	3.4	0.21	0.018	0.22
His^{188}->Gln	11.0	0.32	0.021	0.16
His^{188}->Tyr	6.3	0.09	0.007	0.07

Replacement of His^{42} and His^{188} did not remarkably affect the kinetic constants for both substrate and NADPH. The possibility that either of these His residues may participate in the catalytic action of the enzyme can thus be ruled out by the current investigation. More refined structural data on the enzyme molecule are necessary for further analysis on the precise amino acid residues constituting the catalytic site of aldose reductase by this approach.

Effects of Aldose Reductase Inhibitors on Mutated Enzyme

Aldose reductase inhibitors of diverse chemical structure were evaluated with respect to their effects on the mutated enzymes (Table IV and V). While small effect was detected for Lys^{263}->Met substitution as well as mutation in His^{42} or His^{188} residue, striking increase in K_i for AL1576 and sorbinil was demonstrated on Lys^{263}->Glu substitution. The inhibitor constants for these inhibitors on Lys^{263}->Arg substitution were moderately affected. Among the inhibitors, K_i values for tolrestat was least affected by Lys^{263} replacement. The result that Lys^{263}->Glu substitution significantly deteriorates affinity of sorbinil and AL1576 for enzyme indicates that the binding or associating sites of both inhibitors on enzyme molecule are closely correlated with the physico-chemical property of this Lys residue. Minor effect of Lys^{263} mutation on the K_i for tolrestat, on the other hand, suggests that the associating site for this inhibitor on the enzyme may not reside in the vicinity of the Lys^{263} residue as those for sorbinil and AL1576. These findings illustrate significant difference in the

TABLE V

Effect of Lys^{263} mutation on inhibitor constants for aldose reductase

	K_i (μM)		
	Sorbinil	AL1576	Tolrestat
Wild-type	5	0.30	0.067
Lys^{263}->Met	3	0.55	0.043
Lys^{263}->Glu	175	135	0.77
Lys^{263}->Arg	54	1.9	0.32

inhibitor associating sites for aldose reductase inhibitors depending upon the structure of inhibitors.

In conclusion, our present investigation using site-directed mutagenesis unequivocally demonstrates essential role of Lys^{263} in the enzyme reaction of aldose reductase. The kinetic data obtained in our study are highly indicative of the electrostatic interaction of this Lys residue with the substrate, rather than primary interaction with the cofactor NADPH. Results on inhibitor constants for diverse aldose reductase inhibitors against mutated enzyme revealed significant variation in the associating sites for respective inhibitors on the enzyme molecule. Present study hence provides a novel approach to elucidate possible amino acid residues constituting not only the functional domains participating in the enzyme reaction but also inhibitor associating sites of human aldose reductase, which is known to play a key role in the development of various diabetic complications.

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