Identification of arginine-700 as the residue that binds the C-5 carboxyl group of 2-oxoglutarate in human lysyl hydroxylase 1

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Abstract Lysyl hydroxylase catalyzes the formation of hydroxylysine in collagens by a reaction that involves oxidative decarboxylation of 2-oxoglutarate. Its binding site can be divided into two main subsites: subsite I consists of a positively charged side-chain which binds the C-5 carboxyl group, while subsite II consists of two coordination sites of the enzyme-bound Fe^{2+} and is chelated by the C-1-C-2 moiety. In order to identify subsite I, we converted Arg-697, Arg-700 and Ser-705 individually to alanine and Arg-700 also to lysine, and expressed the mutant enzymes in insect cells. Arg-700-Ala inactivated lysyl hydroxylase completely, whereas Arg-697-Ala and Ser-723-Ala had only a relatively minor effect. Arg-700-Lys produced 93% inactivation under standard assay conditions, the main effect being a 10-fold increase in the K_m for 2-oxoglutarate, whereas the V_{max} was unchanged. Arg-700 thus provides the positively charged residue that binds the C-5 carboxyl group of 2-oxoglutarate, whereas Ser-705 appears to be of no functional significance in this binding.

Key words: Lysyl hydroxylase; 2-Oxoglutarate; Dioxygenase; Catalytic site; Collagen

1. Introduction

Lysyl hydroxylase (EC 1.14.11.49), a homodimer with a subunit molecular weight of about 82 000, catalyzes the formation of hydroxylysine in collagens and more than 10 additional proteins with collagen-like sequences by the hydroxylation of lysine residues in -X-Lys-Gly triplets. The reaction requires Fe^{2+}, 2-oxoglutarate, O_2 and ascorbate and involves oxidative decarboxylation of 2-oxoglutarate (for a recent review, see [1]). Two isoenzymes, termed lysyl hydroxylase 2 [2] and 3 [3,4], have recently been cloned from human sources and characterized, and the previously known main isoenzyme is now correspondingly termed lysyl hydroxylase 1. The lysyl hydroxylase reaction plays an important role in the synthesis of all collagens, as the hydroxylysine residues serve as attachment sites for galactose or glucosylgalactose and are essential for the stability of the intermolecular collagen crosslinks [1]. This critical function of hydroxylysine is demonstrated by changes in the mechanical properties of certain tissues in patients with the type VI variant of the Ehlers-Danlos syndrome [5,6], which is due to various mutations in the gene for lysyl hydroxylase 1 [7–12].

The catalytic cycle of lysyl hydroxylase can be divided into two half-reactions: initial generation of the hydroxylating species and its subsequent utilization for hydroxylysine formation [1,13]. Recent site-directed mutagenesis studies have indicated that the three ligands needed for the binding of Fe^{2+} to the catalytic site in human lysyl hydroxylase 1 [1,13] are His-638, Asp-640 and His-690 (numbering begins with the first residue in the processed polypeptide) [14]. The 2-oxoglutarate binding site can be divided into two main subsites: subsite I is assumed to consist of a positively charged side-chain of the enzyme which binds the C-5 carboxyl group of 2-oxoglutarate, while subsite II consists of two *cis*-positioned coordination sites of the enzyme-bound Fe^{2+} and is chelated by the C-1-C-2 moiety [1,13]. Recent site-directed mutagenesis studies on prolyl 4-hydroxylase, another collagen hydroxylating enzyme with a similar reaction mechanism but essentially no amino acid sequence similarity to lysyl hydroxylase [1,13,15], have demonstrated that the residue that binds the C-5 carboxyl group of 2-oxoglutarate in the α subunit of that enzyme is a lysine in position +10 with respect to the histidine that corresponds to His-690 in lysyl hydroxylase 1 [16].

The aim of the present work was to identify the positively charged residue that binds the C-5 carboxyl group of 2-oxoglutarate in recombinant human lysyl hydroxylase 1. Recent structural studies on a related enzyme, isopenicillin *N*-synthase, have indicated that the carboxyl group of valine in its tripeptide substrate becomes bound to Arg-279 and Ser-281, while the three Fe^{2+} binding ligands are His-214, Asp-216 and His-270 [17]. We therefore also studied whether Ser-705, the only serine present between His-690 and the C-terminus (residue 709), has any role in the binding of the C-5 group of 2-oxoglutarate.

2. Materials and methods

2.1. Site-directed mutagenesis and generation of recombinant baculoviruses

The codons for Arg-697, Arg-700 and Ser-705 in the cDNA for human lysyl hydroxylase 1 [18] were converted individually to codons for alanine, and that for Arg-700 also to one for lysine. The mutagenesis steps were performed in a pBluescript vector (Stratagene) containing the full-length cDNA. The mutagenesis was carried out using an oligonucleotide-directed in vitro system based on the unique site elimination procedure (Pharmacia Biotech Inc.), after which the plasmid was digested with *Eag*I and *Eco*RI. The resulting fragment was cloned to *Eag*I-*Eco*RI-digested baculovirus transfer vector pVL1392 and the sequences were verified by dideoxynucleotide sequencing [19].

The recombinant baculovirus transfer vectors were co-transfected into *Spodoptera frugiperda* Sf9 cells with a modified *Autographa californica* nuclear polyhedrosis virus DNA (Pharmingen) by calcium phosphate transfection, and the recombinant viruses were selected [20].

2.2. Analysis of recombinant proteins in insect cells

High-Five cells (Invitrogen) were cultured as monolayers in TNM-FH medium (Sigma) supplemented with 10% fetal bovine serum (BioClear) at 27°C. Cells seeded at a density of 5×10^6/plate were infected at a multiplicity of 5 with any of the viruses encoding the wild-type lysyl hydroxylase 1 [14] or a mutant type. The cells were harvested 72 h

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LH1	H	E	G	L	·	P	T	·	·	·	T	R	G	·	T	R	Y	I	A	V	S	F	V	D	P	term
LH2	H	E	G	L	·	P	V	·	·	·	K	N	G	·	T	R	Y	I	A	V	S	F	I	D	P	term
LH3	H	E	C	L	·	P	T	·	·	·	T	W	G	·	T	R	Y	I	M	V	S	F	V	D	P	term
α4PH1	H	A	A	C	·	P	V	·	·	·	L	V	G	·	N	K	·	·	W	V	S	N	K	W	L	
α4PH2	H	A	A	C	·	P	V	·	·	·	L	V	G	·	C	K	·	·	W	V	S	N	K	W	F	
ACCO	H	R	V	I	·	Q	T	·	·	·	·	D	G	·	T	R	·	·	·	M	S	L	A	S	F	
F3OH	H	Q	A	V	·	N	S	·	·	·	·	E	C	·	S	R	·	·	·	L	S	I	A	T	F	
H6H	H	R	V	V	T	D	P	·	·	·	T	R	D	·	R	R	·	·	·	V	S	I	A	T	L	
FOLS	H	R	T	T	V	N	·	·	·	·	K	D	K	·	T	R	·	·	·	M	S	W	P	V	F	
GC20	H	R	A	V	U	N	·	·	·	·	S	M	N	·	A	R	·	·	·	K	S	L	A	F	F	
DAOCS	H	R	V	A	A	P	R	R	D	Q	I	A	G	S	S	R	·	·	·	T	S	S	V	F	F	
DAOC/DAOCS	H	R	V	K	S	P	E	R	D	Q	R	V	G	S	S	R	·	·	·	T	S	S	V	F	F	
IPNS	H	R	V	K	W	·	·	·	·	·	V	N	A	·	E	R	·	·	·	Q	S	L	P	F	F	

Fig. 1. Comparison of the amino acid sequence from His-690 to the C-terminus of human lysyl hydroxylase 1 with corresponding sequences in other human lysyl hydroxylase isoenzymes, other 2-oxoglutarate dioxygenases and isopenicillin *N*-synthase. Results are shown for the three human lysyl hydroxylase isoenzymes (LH1–3) [2–4,18], two human prolyl 4-hydroxylase α subunit isoforms (α4PH1 and 2) [31,33], 1-aminocyclopropane-1-carboxylate oxidase (ACCO) from tomato [23], flavanone 3-β-hydroxylase (F3OH) from *Zea mays* [24], hyoscyamine 6-β-hydroxylase (H6H) from henbane [25], flavanol synthase (FOLS) from *Petunia hybrida* [26], gibberellin C-20 oxidase (GC20) from *Cucurbita maxima* [27], deacetoxycephalosporin *C*-synthase (DAOCS) from *Streptomyces clavuligerus* [28], deacetoxycephalosporin *C*-synthase/deacetylcephalosporin *C*-synthase (DAOC/DACS) from *Cephalosporium acremonium* [29], and isopenicillin *N*-synthase (IPNS) from *Aspergillus nidulans* [30]. Gaps (·) are introduced for alignment. The positions of His-690, Arg-700 and Ser-705 in the human lysyl hydroxylase 1 sequence are indicated by asterisks (*) and that of Arg-697 by a dot (•).

after infection, washed with a solution of 0.15 M NaCl and 0.02 M phosphate, pH 7.4, and frozen at −20°C. The frozen cell pellets were suspended and homogenized in a 1% Nonidet P-40, 0.1 M glycine and 0.02 M Tris buffer, pH 7.8, and centrifuged at 10 000×*g* for 10 min. The insoluble pellets were further homogenized in a 50% glycerol, 0.6 M NaCl, 1% Nonidet P40, 0.1 M glycine, 100 μM dithiothreitol and 0.06 M Tris buffer, pH 7.8 (glycerol buffer), incubated on ice for 30–60 min, and centrifuged at 10 000×*g* for 20 min [14]. Aliquots of the supernatants were analyzed by 8% SDS-PAGE under reducing conditions and assayed for lysyl hydroxylase activity.

2.3. Other assays

Lysyl hydroxylase activity was assayed by a method based on the hydroxylation-coupled decarboxylation of 2-oxo-[1-^{14}C]glutarate [21]. K_m values were determined as described previously [22] and protein concentrations with a Bio-Rad protein assay kit (Bio-Rad). The levels of expression of the wild-type and mutant lysyl hydroxylases were compared by densitometry of the Coomassie-stained bands in SDS-PAGE using a BioImage instrument (BioImage, Millipore).

3. Results and discussion

The sequences of the three human lysyl hydroxylase isoenzymes between His-690 (numbered according to the human lysyl hydroxylase 1 sequence) and the C-terminus (residue 709) show a high degree of identity. Two arginine residues are present in this region, but no lysine or histidine (Fig. 1). The residue corresponding to Arg-700 is conserved in the sequences of all lysyl hydroxylases [1–4,18], in many 2-oxoglutarate dioxygenases [23–29] and in isopenicillin *N*-synthase [30] (Fig. 1). This position is occupied in all prolyl 4-hydroxylases [1,31–33] by the lysine that has been shown by site-directed mutagenesis studies to bind the C-5 carboxyl group of 2-oxoglutarate [16] (Fig. 1). Arg-697 is not conserved in chick lysyl hydroxylase 1 [34] or in human lysyl hydroxylases 2 and 3 (Fig. 1), and was thus not likely to be involved in the binding of 2-oxoglutarate and could be used as a control residue in the mutagenesis studies. The only serine present between His-690 and the C-terminus is residue 705, which is conserved in all forms of lysyl hydroxylase. If gaps are introduced into the sequences, this residue can be aligned with the serine that participates in the binding of the carboxyl group of valine [17] in the tripeptide substrate of isopenicillin *N*-synthase (Fig. 1).

To study the possible functional significance of Arg-700 and Ser-705, these two residues and Arg-697 were converted individually to alanine, and after preliminary experiments, Arg-700 was also converted to lysine. All the mutant polypeptides were expressed in High-Five insect cells. The cells were harvested 48 h after infection, homogenized in a buffer containing 1% Nonidet P-40 and centrifuged. The cell pellet was homogenized further in a buffer containing 50% glycerol [14], and the proteins soluble in the glycerol buffer were analyzed by SDS-PAGE performed under reducing conditions. Aliquots of the glycerol buffer extract were also used to assay lysyl hydroxylase activity and protein content.

No differences in mobility were found between the various mutant lysyl hydroxylase polypeptides in SDS-PAGE, and only very minor ones were found in the amounts of the polypeptides in the glycerol buffer extracts as judged from the intensities of the Coomassie-stained bands corresponding to the variably glycosylated lysyl hydroxylase polypeptides (Fig.

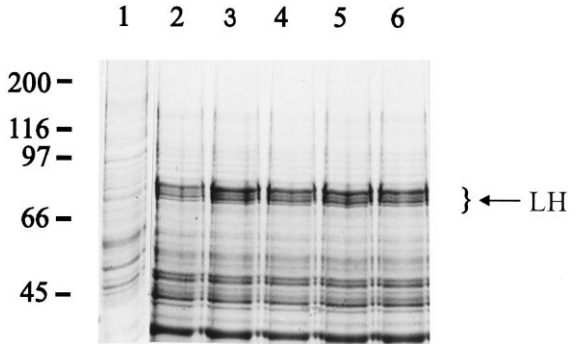

Fig. 2. SDS-PAGE analysis of the wild-type and various mutant human lysyl hydroxylase 1 polypeptides expressed in insect cells. The infected cells were homogenized and extracted with the glycerol buffer. The samples were analyzed by 8% SDS-PAGE under reducing conditions followed by Coomassie staining. Lane 1, extract from non-infected High-Five cells; lane 2, extract from cells infected with the baculovirus encoding wild-type lysyl hydroxylase 1; lanes 3–6, extracts from cells infected with baculoviruses encoding the mutant lysyl hydroxylases Arg-697-Ala (lane 3), Arg-700-Ala (lane 4), Ser-705-Ala (lane 5) and Arg-700-Lys (lane 6). The arrow indicates the positions of the variably glycosylated lysyl hydroxylase (LH) polypeptides.

Table 1
Lysyl hydroxylase activity in glycerol buffer extracts of cells expressing various arginine to alanine or lysine or serine to alanine mutant lysyl hydroxylases

Experiment and enzyme expressed	2-Oxoglutarate concentration (μM)	Lysyl hydroxylase activity[a]	
		dpm/100 μg	%
Experiments 1–4			
Wild-type	100	3590 ± 360	100
Arg-697-Ala	100	2920 ± 540	81
Arg-700-Ala	100	< 50	< 2
Arg-700-Lys	100	240 ± 160	7
Ser-705-Ala	100	2390 ± 310	67
Experiments 5–6			
Wild-type	300	4580 ± 20	100
Arg-700-Lys	300	660 ± 100	14

[a]Values are given in dpm/100 μg of extractable cell protein in the glycerol buffer extract, mean ± S.D., and in percent of the value obtained with the wild-type virus. The values have been corrected further for minor differences in expression levels compared with the wild-type enzyme by densitometry of the Coomassie-stained bands in SDS-PAGE.

2). These bands were studied by densitometry in each experiment, and the amounts of the various mutants were calculated as percentages of the amount of the wild-type enzyme. These percentages were then used to correct the enzyme activities of the various mutants to the minor differences in expression levels.

Mutation of Arg-700 to alanine was found to inactivate the lysyl hydroxylase completely, whereas mutation of Arg-697 to alanine had only a minor effect (Table 1). Mutation of Ser-723 to alanine had a slightly larger effect on enzyme activity than the Arg-697-Ala mutation, but it was still very minor relative to that of the Arg-700-Ala mutation (Table 1). The Arg-700-Lys mutant enzyme was not completely inactive, but the activity level obtained in assays using 100 μM 2-oxoglutarate was only about 7% of that of the wild-type enzyme (Table 1). When the 2-oxoglutarate concentration in the assay reaction was increased to 300 μM, the relative activity of the Arg-700-Lys mutant increased to about 14% (Table 1) suggesting impaired binding of 2-oxoglutarate.

To study the effects of the Arg-700-Lys and Ser-705-Ala mutations in more detail, K_m values for 2-oxoglutarate were determined for the wild-type enzyme and these mutants with the glycerol buffer extracts from cells expressing these polypeptides as enzyme sources. The K_m of the Arg-700-Lys mutant enzyme was found to be markedly increased, about 10-fold, whereas the K_m of the Ser-705-Ala mutant was identical to that of the wild-type enzyme (Table 2). The V_{max} obtained with the Arg-700-Lys enzyme was the same as with the wild-type enzyme (details not shown).

Our data thus strongly suggest that Arg-700 is the positively charged residue that binds the C5 carboxyl group of 2-oxoglutarate in human lysyl hydroxylase 1, whereas Ser-705 appears to be of no functional significance in this binding. An arginine corresponding to Arg-700 is conserved in the sequences of almost all 2-oxoglutarate dioxygenases studied so far (Fig. 1) and is thus likely to have a similar function in almost all members of this enzyme group. Surprisingly, however, the corresponding residue in the α subunits of prolyl 4-hydroxylases from all sources studied is lysine [1,31–33]. When this lysine was converted to arginine by site-directed mutagenesis, the K_m for 2-oxoglutarate increased about 15-fold with no change in the V_{max} of the reaction [16]. The present data indicate that when Arg-700 in lysyl hydroxylase 1 was converted to lysine, the K_m for 2-oxoglutarate also increased markedly. Thus the two collagen hydroxylases clearly have a different requirement with respect to the residue that binds the C5-carboxyl group of 2-oxoglutarate.

Table 2
K_m values of the wild-type and Arg-700-Lys and Ser-705-Ala mutant lysyl hydroxylases for 2-oxoglutarate

Enzyme	K_m[a] (μM)
Wild-type	100 ± 25
Arg-718-Lys	1000 ± 160
Ser07230Ala	100

All K_m values were determined using glycerol buffer extracts of cells expressing either the wild-type or mutant lysyl hydroxylases as sources of the enzyme.
[a]The values are given as the mean ± S.D., $n = 8$ for the wild-type lysyl hydroxylase, $n = 11$ for Arg-700-Lys, and $n = 1$ for Ser-705-Ala.

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