

# Characterization of the iron- and 2-oxoglutarate-binding sites of human prolyl 4-hydroxylase

Johanna Myllyharju and Kari I. Kivirikko<sup>1</sup>

Collagen Research Unit, Biocenter and Department of Medical Biochemistry, University of Oulu, Kajaanintie 52 A, FIN-90220 Oulu, Finland

<sup>1</sup>Corresponding author

**Prolyl 4-hydroxylase (EC 1.14.11.2), an  $\alpha_2\beta_2$  tetramer, catalyzes the formation of 4-hydroxyproline in collagens. We converted 16 residues in the human  $\alpha$  subunit individually to other amino acids, and expressed the mutant polypeptides together with the wild-type  $\beta$  subunit in insect cells. Asp414Ala and Asp414Asn inactivated the enzyme completely, whereas Asp414Glu increased the  $K_m$  for  $\text{Fe}^{2+}$  15-fold and that for 2-oxoglutarate 5-fold. His412Glu, His483Glu and His483Arg inactivated the tetramer completely, as did Lys493Ala and Lys493His, whereas Lys493Arg increased the  $K_m$  for 2-oxoglutarate 15-fold. His501Arg, His501Lys, His501Asn and His501Gln reduced the enzyme activity by 85–95%; all these mutations increased the  $K_m$  for 2-oxoglutarate 2- to 3-fold and enhanced the rate of uncoupled decarboxylation of 2-oxoglutarate as a percentage of the rate of the complete reaction up to 12-fold. These and other data indicate that His412, Asp414 and His483 provide the three ligands required for the binding of  $\text{Fe}^{2+}$  to a catalytic site, while Lys493 provides the residue required for binding of the C-5 carboxyl group of 2-oxoglutarate. His501 is an additional critical residue at the catalytic site, probably being involved in both the binding of the C-1 carboxyl group of 2-oxoglutarate and the decarboxylation of this cosubstrate.**

**Keywords:** catalytic site/collagen/dioxygenases/non-heme iron centers/2-oxoglutarate-dependent enzymes

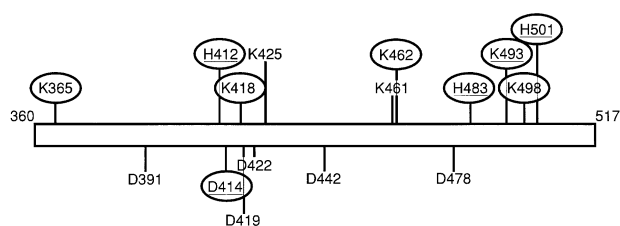
## Introduction

Prolyl 4-hydroxylase (EC 1.14.11.2) catalyzes the hydroxylation of proline in -X-Pro-Gly- sequences in collagens and related proteins. This co-translational and post-translational modification plays a central role in the synthesis of all collagens, as 4-hydroxyproline residues are essential for the folding of the newly synthesized collagen polypeptide chains into triple-helical molecules. The hydroxylation requires  $\text{Fe}^{2+}$ , 2-oxoglutarate,  $\text{O}_2$  and ascorbate and involves an oxidative decarboxylation of 2-oxoglutarate (for reviews, see Kivirikko *et al.*, 1989, 1992; Prockop and Kivirikko, 1995).

Prolyl 4-hydroxylase from vertebrates is an  $\alpha_2\beta_2$  tetramer, in which the  $\beta$  subunits are identical to the protein disulfide isomerase (PDI, EC 5.3.4.1, Koivu *et al.*, 1987; Pihlajaniemi *et al.*, 1987; Parkkonen *et al.*, 1988) and the  $\alpha$  subunits contribute to most parts of the two catalytic

sites (Kivirikko *et al.*, 1989, 1992). A catalytic site appears to comprise a set of separate locations for the binding of the various co-substrates and the peptide substrate. The  $\text{Fe}^{2+}$  is probably coordinated with the  $\alpha$  subunit by three side chains (Hanauske-Abel and Günzler, 1982; Kivirikko *et al.*, 1989, 1992). The 2-oxoglutarate-binding site can be divided into at least two distinct subsites: subsite I probably consists of a positively charged side chain of the  $\alpha$  subunit, which binds the C-5 carboxyl group of the 2-oxoglutarate, while subsite II consists of two *cis*-positioned coordination sites of the enzyme-bound  $\text{Fe}^{2+}$  and is chelated by the C-1–C-2 moiety (Hanauske-Abel and Günzler, 1982; Majamaa *et al.*, 1984; Kivirikko *et al.*, 1989, 1992). The  $\text{O}_2$  is thought to be bound to the  $\text{Fe}^{2+}$  end-on in an axial position, and the subsequent decarboxylation of 2-oxoglutarate is thought to lead to the formation of ferryl ion, which hydroxylates a proline residue in the second half-reaction of the catalytic cycle (Hanauske-Abel and Günzler, 1982; Kivirikko *et al.*, 1989, 1992; Hanauske-Abel, 1991). Ascorbate acts as an alternative oxygen acceptor in the uncoupled decarboxylation cycles, in which 2-oxoglutarate is decarboxylated without subsequent hydroxylation of the peptide substrate (de Jong and Kemp, 1984; Myllylä *et al.*, 1984; Majamaa *et al.*, 1986).

A search for conserved residues within the sequences of several 2-oxoglutarate dioxygenases and a related dioxygenase, isopenicillin *N* synthase, indicated weak homology within two histidine-containing motifs, His-1 and His-2, spaced ~50–70 amino acids apart (Myllylä *et al.*, 1992), the histidines concerned in the human  $\alpha$  subunit sequence (Helaakoski *et al.*, 1989) being His412 and His483. Site-directed mutagenesis indicated that conversion of either of these histidines to serine inactivated the enzyme completely, while mutation of one additional histidine, His501, inactivated it by ~96% (Lamberg *et al.*, 1995). As analyses of the related enzyme, isopenicillin *N* synthase, by a variety of techniques had suggested that all three  $\text{Fe}^{2+}$ -binding ligands in that enzyme may be histidines (Jiang *et al.*, 1991; Ming *et al.*, 1991; Randall *et al.*, 1993), it initially was considered possible that these three histidines might provide the three  $\text{Fe}^{2+}$ -binding ligands in prolyl 4-hydroxylase, even though the His501Ser mutation did not inactivate the enzyme completely (Lamberg *et al.*, 1995). Recent determination of the crystal structure of isopenicillin *N* synthase has indicated, however, that although two of the  $\text{Fe}^{2+}$ -binding ligands are histidines, corresponding to those in the His-1 and His-2 motifs, the third is an aspartate present in position +2 with respect to the histidine in the His-1 motif (Roach *et al.*, 1995). A corresponding aspartate residue is found in the  $\alpha$  subunits of all prolyl 4-hydroxylases studied (Helaakoski *et al.*, 1989, 1995; Bassuk *et al.*, 1989; Veijola *et al.*, 1994).



**Fig. 1.** Schematic representation of the conserved C-terminal region (residues 387–513) of the type I  $\alpha$  subunit of human prolyl 4-hydroxylase. Aspartate and lysine residues present in or near the conserved C-terminal region are shown, and it is these that were subjected to mutagenesis. Three histidine residues previously found to be critical for the catalytic activity of prolyl 4-hydroxylase (Lamberg *et al.*, 1995) are also shown. Numbering of the amino acids begins with the first residue in the processed  $\alpha$  subunit, and conserved residues are circled. The  $\text{Fe}^{2+}$ -binding residues identified here or previously (Lamberg *et al.*, 1995), i.e. His412, Asp414 and His483, the 2-oxoglutarate-binding lysine 493 and the additional critical histidine His501 are underlined.

The aim of the present work was to identify in detail all four critical residues involved in the binding of  $\text{Fe}^{2+}$  (three ligands) and 2-oxoglutarate (subsite I) to the catalytic site of human type I prolyl 4-hydroxylase (Helaakoski *et al.*, 1989) and to determine whether some of these can be replaced by other residues. We also attempted to determine whether His501 is involved in any of these functions.

## Results

### **Aspartate 414 is an essential residue but can be partly replaced by glutamate**

Six aspartate residues were converted individually to alanine (Figure 1). These represented all six aspartates present within the highly conserved 127 amino acid region (residues 387–513) close to the COOH-terminus of the prolyl 4-hydroxylase  $\alpha$  subunit. Asp414 (circled in Figure 1) is the only residue among these that is conserved between the human and *Caenorhabditis elegans*  $\alpha$  subunits (Helaakoski *et al.*, 1989; Veijola *et al.*, 1994). All the mutant  $\alpha$  subunits were expressed in Sf9 cells together with the wild-type PDI/ $\beta$  polypeptide. The cells were harvested 72 h after infection, homogenized in a buffer containing Triton X-100 and centrifuged, and the remaining pellets were solubilized in 1% SDS. No differences were found in the mobilities of the various mutant  $\alpha$  subunits in SDS-PAGE analysis (data not shown). The Triton X-100-soluble proteins were then analyzed by non-denaturing PAGE and Coomassie staining and assayed for prolyl 4-hydroxylase activity by a method based on the hydroxylation-coupled decarboxylation of 2-oxo[1- $^{14}\text{C}$ ]glutarate. The Coomassie-stained bands corresponding to the enzyme tetramer 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 differences in the expression levels. In almost all cases this correction was less than  $\pm 20\%$ .

Five of the six mutant  $\alpha$  subunits formed an enzyme tetramer with the PDI/ $\beta$  polypeptide (Figure 2A). Asp478Ala was the only mutant that did not form a tetramer, indicating that although this position can be

occupied by aspartate, or by asparagine in the *C.elegans*  $\alpha$  subunit (Veijola *et al.*, 1994), alanine in this position will prevent tetramer formation. As the intention here was to characterize the catalytic sites of the enzyme rather than its tetramer assembly, this aspect was not studied any further.

Only one aspartate to alanine mutation was found to have a marked effect on prolyl 4-hydroxylase activity in the enzyme tetramer. Mutation of the conserved Asp414 (underlined in Figure 1), the residue corresponding to the  $\text{Fe}^{2+}$ -binding aspartate in isopenicillin *N* synthase, inactivated the enzyme completely (Table I). The Asp391Ala mutation had no effect on enzyme activity, while Asp422Ala and Asp442Ala reduced it by about half and Asp419Ala increased it by  $\sim 20\%$  (Table I). The  $K_m$  values of the Asp419Ala, Asp422Ala and Asp442Ala mutant prolyl 4-hydroxylases for  $\text{Fe}^{2+}$  and 2-oxoglutarate were identical to those of the wild-type enzyme (details not shown).

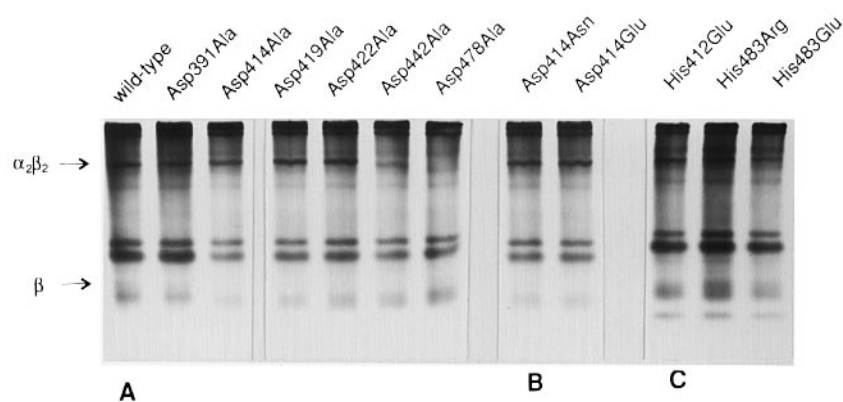
When Asp414 was replaced with asparagine and glutamate, both mutant  $\alpha$  subunits formed an enzyme tetramer with the PDI/ $\beta$  polypeptide (Figure 2B). The mutation Asp414Asn eliminated the enzyme activity completely, whereas Asp414Glu reduced it to  $\sim 15\%$  (Table I). The  $K_m$  of the Asp414Glu mutant prolyl 4-hydroxylase for  $\text{Fe}^{2+}$  was markedly increased relative to the wild-type enzyme,  $\sim 15$ -fold (Table II). The  $K_m$  for 2-oxoglutarate was also increased, although not as markedly,  $\sim 5$ -fold (Table II). The  $V_{\max}$  determined from the kinetic plots was identical to that obtained with the wild-type enzyme (details not shown).

### **Histidines 412 and 483 cannot be replaced by a negatively or positively charged residue**

Mutation of the two histidines present in the His-1 and His-2 motifs of prolyl 4-hydroxylase, His412 and His483 (underlined in Figure 1), to serine has been shown previously to inactivate the enzyme completely (Lamberg *et al.*, 1995). Site-directed mutagenesis of bovine aspartyl (asparaginy)  $\beta$ -hydroxylase has shown that substitution of the histidine present in the His-2 motif of that enzyme with a negatively charged amino acid results in 10–20% residual activity, whereas substitution with an uncharged or a positively charged residue results in complete inactivation (Jia *et al.*, 1994; McGinnis *et al.*, 1996). We therefore converted the His412 and His483 of the prolyl 4-hydroxylase  $\alpha$  subunit individually to glutamate, and His483 also to arginine. The mutant  $\alpha$  subunits were then expressed together with the PDI/ $\beta$  polypeptide in Sf9 cells and analyzed as above. All three mutant  $\alpha$  subunits formed enzyme tetramers with the PDI/ $\beta$  polypeptide, as shown by the presence of a Coomassie-stained band in non-denaturing PAGE (Figure 2C). None of the His412Glu, His483Arg and His483Glu mutant prolyl 4-hydroxylases had any enzyme activity, however (Table I).

### **Lysine 493 is involved in the binding of 2-oxoglutarate**

Attempts to model the  $\text{Fe}^{2+}$ -binding region of the human  $\alpha$  subunit sequence suggested that the residue that ionically binds the C-5 carboxyl group of the 2-oxoglutarate (subsite I) may be Lys493 or Lys498 (A.Haataja and A.Koskinen, unpublished data). Seven lysine residues, five of which



**Fig. 2.** Non-denaturing PAGE analysis of prolyl 4-hydroxylase tetramer formation from the wild-type or various aspartate (**A** and **B**) or histidine (**C**) mutant human  $\alpha$  subunits and the PDI/ $\beta$  polypeptide expressed in insect cells by means of baculovirus vectors. The samples were extracted with a buffer containing 0.1% Triton X-100 and analyzed by non-denaturing PAGE and Coomassie staining. Mutant enzymes are indicated by the numbers of the  $\alpha$  subunit residues converted. The arrows indicate the positions of the enzyme tetramer ( $\alpha_2\beta_2$ ) and the non-associated PDI/ $\beta$  polypeptide ( $\beta$ ). The non-associated  $\alpha$  subunit is insoluble in 0.1% Triton X-100.

**Table I.** Activity of certain aspartate and histidine mutant prolyl 4-hydroxylases

Enzyme <sup>a</sup>	Prolyl 4-hydroxylase activity	
	(d.p.m./10 $\mu$ g)	(%)
Non-infected control	<50	<1
Wild-type	7460 $\pm$ 980	100 $\pm$ 13
Asp391Ala	7580 $\pm$ 1050	102 $\pm$ 14
Asp414Ala	<50	<1
Asp419Ala	9290 $\pm$ 280	124 $\pm$ 4
Asp422Ala	4180 $\pm$ 330	56 $\pm$ 5
Asp442Ala	3530 $\pm$ 910	48 $\pm$ 12
Asp414Asn	<50	<1
Asp414Glu	1100 $\pm$ 180	15 $\pm$ 2
His412Glu	<50	<1
His483Arg	<50	<1
His483Glu	<50	<1

Values are given in d.p.m./10  $\mu$ g of extractable cell protein, mean  $\pm$  SD for at least four experiments, and as percentages  $\pm$  SD of the value obtained with the wild-type enzyme. All values are corrected by densitometry of the tetramer band seen in non-denaturing PAGE analysis for minor differences in the amounts of the enzyme tetramer formed.

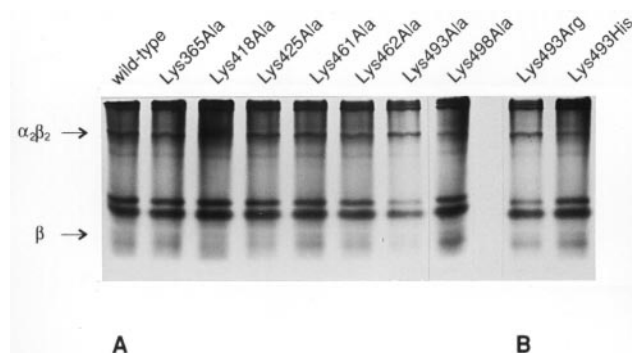
<sup>a</sup>Mutant enzymes are indicated by the numbers of the  $\alpha$  subunit residues converted.

**Table II.**  $K_m$  values of the wild-type and certain mutant prolyl 4-hydroxylases for co-substrates and the peptide substrate

Enzyme <sup>a</sup>	$K_m$ ( $\mu$ M)			
	Fe <sup>2+</sup>	2-Oxoglutarate	Ascorbate	(Pro-Pro-Gly) <sub>10</sub>
Wild-type	2	20	300	15
Asp414Glu	30	100	270	15
Lys493Arg	2	300	300	24
His501Arg	2	40		20
His501Asn	2	60		22
His501Gln	2	60		
His501Lys	2	65		24
His501Ser	2	60		22

All  $K_m$  values were determined using Triton X-100 extracts from cells expressing either the wild-type or mutant prolyl 4-hydroxylase as sources of the enzyme.

<sup>a</sup>Mutant enzymes are indicated by the numbers of the  $\alpha$  subunit residues converted.



**Fig. 3.** Non-denaturing PAGE analysis of prolyl 4-hydroxylase tetramer formation from the wild-type or various lysine (**A** and **B**) mutant human  $\alpha$  subunits and the PDI/ $\beta$  polypeptide expressed in insect cells by means of baculovirus vectors. Experimental details are as in Figure 2. Mutant enzymes are indicated by the numbers of the  $\alpha$  subunit residues converted. The arrows indicate the positions of the enzyme tetramer ( $\alpha_2\beta_2$ ) and the non-associated PDI/ $\beta$  polypeptide ( $\beta$ ).

are conserved between the human (Helaakoski *et al.*, 1989) and *C.elegans* (Veijola *et al.*, 1994)  $\alpha$  subunit sequences (circled in Figure 1), were therefore converted individually to alanine, and the mutant polypeptides were expressed in Sf9 cells together with the wild-type PDI/ $\beta$  polypeptide. The Triton X-100-soluble proteins were then used for the analyses as above. None of the mutations was found to inhibit tetramer assembly (Figure 3A), and only minor differences were found in the amounts of tetramer formed, as judged from the intensities of the Coomassie-stained bands in non-denaturing PAGE (details not shown). Mutation of the conserved Lys493 (underlined in Figure 1) inactivated the enzyme completely (Table III), while mutation of the other conserved lysines either reduced the enzyme activity by ~15–30% (Lys365Ala, Lys418Ala and Lys498Ala), or increased it by ~20% (Lys462Ala). Mutation of the non-conserved Lys425 had no effect on enzyme activity, but mutation of the other non-conserved Lys461 increased it by ~30% (Table III). As in the case of the aspartate mutations, none of these six lysine mutations with slight effects on enzyme activity altered the  $K_m$  values for Fe<sup>2+</sup> or 2-oxoglutarate (details not shown).

To study the role of Lys493 in more detail, this residue

**Table III.** Activity of certain lysine mutant prolyl 4-hydroxylases

Enzyme <sup>a</sup>	Prolyl 4-hydroxylase activity	
	(d.p.m./10 µg)	(%)
Non-infected control	<50	<1
Wild-type	4870 ± 690	100 ± 12
Lys365Ala	3730 ± 280	76 ± 6
Lys418Ala	4240 ± 810	87 ± 17
Lys425Ala	4570 ± 450	94 ± 9
Lys461Ala	6370 ± 450	131 ± 10
Lys462Ala	5800 ± 1080	119 ± 22
Lys493Ala	<50	<1
Lys498Ala	3310 ± 90	68 ± 2
Lys493Arg	610 ± 260	13 ± 6
Lys493His	<50	<1

Values are given in d.p.m./10 µg of extractable cell protein, mean ± SD for at least four experiments, and as percentages ± SD of the value obtained with the wild-type enzyme. All values are corrected for minor differences in the amounts of enzyme tetramer as in Table I.

<sup>a</sup>Mutant enzymes are indicated by the numbers of the α subunit residues converted.

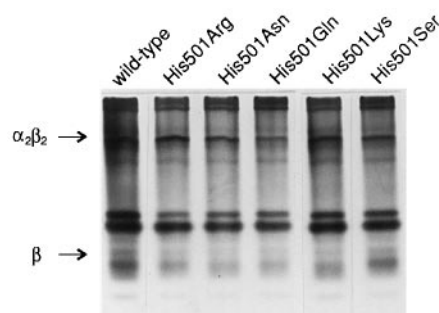
**Table IV.**  $K_i$  values of the wild-type and Lys493Arg mutant prolyl 4-hydroxylases for certain competitive inhibitors with respect to 2-oxoglutarate

	$K_i$ (µM)	
	Wild-type	Lys493Arg
Oxoadipinate	900	3700
Oxaloacetate	100	220
Oxovalerate	4000	4500
Pyridine-2,4-dicarboxylate	2	8
Pyridine-2-carboxylate	25	25

All  $K_i$  values were determined using Triton X-100 extracts from cells expressing either the wild-type or Lys493Arg mutant prolyl 4-hydroxylase as sources of the enzyme.

was also converted to arginine and histidine. Neither mutation had any effect on tetramer assembly (Figure 3B), but Lys493Arg reduced the enzyme activity to ~15% and Lys493His resulted in complete inactivation (Table III). The  $K_m$  values of the Lys493Arg mutant enzyme for  $\text{Fe}^{2+}$ , ascorbate and the peptide substrate showed no differences relative to the wild-type enzyme, but the  $K_m$  for 2-oxoglutarate was markedly increased, ~15-fold (Table II). The  $V_{\max}$  obtained with the Lys493Arg mutant enzyme was the same as with the wild-type enzyme (details not shown).

The  $K_i$  values of the Lys493Arg mutant enzyme for structural analogs of 2-oxoglutarate, oxoadipinate and pyridine 2,4-dicarboxylate, which become bound at both main subsites of the 2-oxoglutarate-binding site (Hanauske-Abel and Günzler, 1982; Majamaa *et al.*, 1984) were also increased, ~4-fold (Table IV), whereas the  $K_i$  values for oxovalerate and pyridine 2-carboxylate, which cannot become bound at subsite I due to the lack of a carboxyl group (Majamaa *et al.*, 1984), were the same as for the wild-type enzyme (Table IV). The  $K_i$  for oxaloacetate, which is shorter than 2-oxoglutarate and therefore reacts poorly at subsite I, was increased ~2-fold (Table IV). The Lys493Arg enzyme, like the wild-type enzyme, was able to utilize 2-oxoadipinate as a co-substrate in

**Fig. 4.** Non-denaturing PAGE analysis of prolyl 4-hydroxylase tetramer formation from wild-type or different H501 mutant human α subunits and the PDI/β polypeptide expressed in insect cells by means of baculovirus vectors. Experimental details are as in Figure 2. The arrows indicate the positions of the enzyme tetramer ( $\alpha_2\beta_2$ ) and the non-associated PDI/β polypeptide ( $\beta$ ).**Table V.** Activity of certain His501 mutant prolyl 4-hydroxylases

Enzyme <sup>a</sup>	Prolyl 4-hydroxylase activity	
	(d.p.m./10 µg)	(%)
Non-infected control	<50	<1
Wild-type	6300 ± 960	100 ± 15
His501Arg	780 ± 70	12 ± 1
His501Asn	380 ± 20	6 ± 0
His501Gln	450 ± 170	7 ± 3
His501Lys	910 ± 170	14 ± 3

Values are given in d.p.m./10 µg of extractable cell protein, mean ± SD for at least four experiments, and as percentages ± SD of the value obtained with the wild-type enzyme. All values are corrected for minor differences in the amounts of enzyme tetramer as in Table I.

<sup>a</sup>Mutant enzymes are indicated by the numbers of the α subunit residues converted.

the hydroxylation of [ $^{14}\text{C}$ ]proline-labeled procollagen substrate (details not shown).

### **Histidine 501 is an important residue but can be partly replaced by arginine or lysine**

Previous site-directed mutagenesis studies have shown that mutation of His501 (underlined in Figure 1) to serine reduces the prolyl 4-hydroxylase activity of the tetramer to ~4%, the  $K_m$  for 2-oxoglutarate being ~3-fold compared with that of the wild-type enzyme (Lamberg *et al.*, 1995). The role of His501 in the catalytic activity was studied further by converting this residue to arginine, asparagine, glutamine and lysine. None of these four mutations was found to inhibit enzyme tetramer formation when the mutant α subunits were expressed in Sf9 cells together with the PDI/β polypeptide (Figure 4). The mutations His501Asn and His501Gln reduced the enzyme activity of the tetramer to ~5%, while His501Arg and His501Lys reduced it to ~10–15% (Table V). The decreases in  $V_{\max}$  values were similar to those observed in the reaction rates measured under standard assay conditions (details not shown). The  $K_m$  values for  $\text{Fe}^{2+}$  and the peptide substrate of the four mutant tetramers were the same as those of the wild-type enzyme, whereas the  $K_m$  values for 2-oxoglutarate were increased ~2- to 3-fold (Table II). The  $K_i$  of the His501Ser mutant enzyme for pyridine 2-carboxylate was likewise increased 2-fold (details not shown).

**Table VI.** Rates of uncoupled decarboxylation of 2-oxoglutarate with certain mutant prolyl 4-hydroxylases

Enzyme <sup>a</sup>	Uncoupled enzyme activity	
	(d.p.m./100 µg)	(% of the complete reaction)
Wild-type	440 ± 100	0.7 ± 0.2
His501Arg	410 ± 110	5.4 ± 1.5
His501Asn	310 ± 40	8.7 ± 1.4
His501Gln	180 ± 30	4.3 ± 0.8
His501Lys	270 ± 70	3.0 ± 0.8
His501Ser	190 ± 40	9.2 ± 1.4
Lys365Ala	300 ± 120	0.6 ± 0.2
Lys462Ala	350 ± 50	0.5 ± 0.1
Lys493Arg	50 ± 30	0.7 ± 0.3
Lys498Ala	430 ± 150	1.0 ± 0.3
Asp414Glu	150 ± 25	1.6 ± 0.3
Asp442Ala	410 ± 280	1.4 ± 0.9

The uncoupled reaction was performed as the complete reaction but without addition of the polypeptide substrate. The values are given in d.p.m./100 µg of extractable cell protein, mean ± SD for at least four experiments, and as percentages ± SD of the value for the complete prolyl 4-hydroxylase reaction obtained in the same experiment.

<sup>a</sup>Mutant enzymes are indicated by the numbers of the α subunit residues converted.

### Rate of uncoupled decarboxylation

Prolyl 4-hydroxylase also catalyzes uncoupled decarboxylation of 2-oxoglutarate (Tuderman *et al.*, 1977; Counts *et al.*, 1978; Rao and Adams, 1978) at a rate which is  $0.7 \pm 0.2\%$  of that of the complete reaction (Table VI). All the mutant prolyl 4-hydroxylases were tested for this rate by performing the enzyme reaction without the polypeptide substrate, and in almost all cases, including those with a markedly decreased rate of the complete reaction, this percentage did not differ significantly from that of the wild-type enzyme (some examples are shown in Table VI). However, the His501 mutants differed distinctly from the wild-type enzyme and all the other mutants, in that the rate of uncoupled decarboxylation decreased much less than the rate of the complete reaction, and thus the percentage of the uncoupled reaction was increased up to ~12-fold, the highest percentages being seen with the His501Asn and His501Ser mutants and the lowest with the His501Lys mutant (Table VI).

Since poly(L-proline), a competitive inhibitor with respect to the polypeptide substrate, enhances the rate of uncoupled decarboxylation (Counts *et al.*, 1978; Myllylä *et al.*, 1984), all the mutants were also tested for uncoupled decarboxylation in the presence of 5 µg/ml of poly(L-proline), mol. wt 7000. The rate of the uncoupled reaction (as d.p.m./100 µg) increased ~1.3- to 1.6-fold with the wild-type enzyme and all the mutants, there being no difference between the His501 mutants and the others (details not shown).

### Discussion

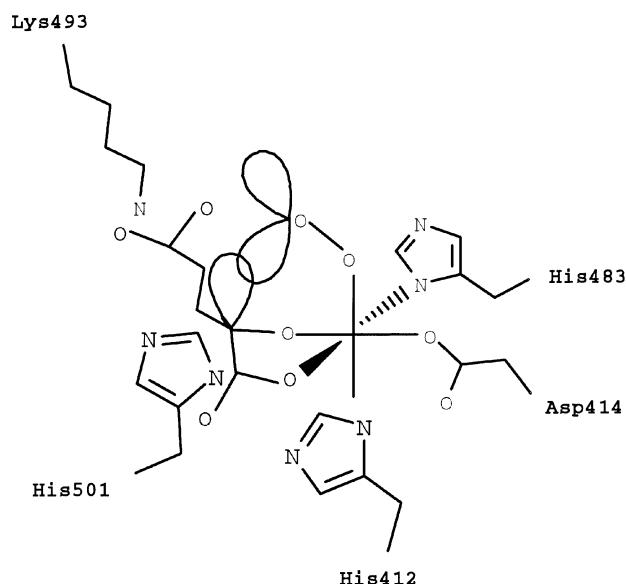
The present data clearly demonstrate that Asp414 is one of the three Fe<sup>2+</sup>-binding ligands in the α subunit of human type I prolyl 4-hydroxylase. Mutation of this residue to alanine or asparagine inactivated the enzyme completely, whereas mutation to glutamate inactivated it by ~85% under standard assay conditions. The marked increase in the  $K_m$  of the Asp414Glu mutant enzyme for

Fe<sup>2+</sup> is in agreement with the proposed function of Asp414 as one of the Fe<sup>2+</sup>-binding ligands, and the increase in that for 2-oxoglutarate, although smaller, is not surprising, as enzyme-bound Fe<sup>2+</sup> constitutes one of the two main binding sites for this co-substrate (Hanauske-Abel and Günzler, 1982; Majamaa *et al.*, 1984). It seems very likely that the longer side chain of glutamate distorts the Fe<sup>2+</sup>-binding site to such an extent that this influences the binding of 2-oxoglutarate to its other main site.

The observation that substitution of glutamate for His412 or His483 or arginine for His483 inactivated prolyl 4-hydroxylase completely differs from recent data on aspartyl (asparaginy) β-hydroxylase, which suggests that the histidine present in the His-2 motif can be replaced by glutamate with retention of partial activity (McGinnis *et al.*, 1996). Due to the complete inactivation, it was not possible to determine whether replacement of either of the two histidines in prolyl 4-hydroxylase by other amino acids increases the  $K_m$  for Fe<sup>2+</sup>. Previous data on the inactivation of prolyl 4-hydroxylase by diethyl pyrocarbonate and prevention of this inactivation by co-substrates of the reaction strongly suggest that histidine residues are functional at the catalytic site, probably at the Fe<sup>2+</sup>-binding site (Myllylä *et al.*, 1992). Furthermore, determination of the crystal structure of isopenicillin N synthase has demonstrated that two of the Fe<sup>2+</sup>-binding ligands in that enzyme are histidines, corresponding to those in the His-1 and His-2 motifs of prolyl 4-hydroxylase, while the third is an aspartate in position +2 with respect to the histidine in the His-1 motif (Roach *et al.*, 1995). It thus seems very likely that the three Fe<sup>2+</sup>-binding ligands in the α subunit of human prolyl 4-hydroxylase are His412, Asp414 and His483 (Figure 5). Recent site-directed mutagenesis studies on a closely related enzyme, lysyl hydroxylase, likewise suggest that the three Fe<sup>2+</sup>-binding ligands in that enzyme are two histidines and an aspartate in the corresponding positions (Pirkanen *et al.*, 1996), although virtually no other amino acid sequence similarities are found between these two enzymes (Myllylä *et al.*, 1991).

Prolyl 4-hydroxylase differs from isopenicillin N synthase in that the latter does not utilize 2-oxoglutarate as a co-substrate. The present data strongly suggest that the residue that ionically binds the C-5 carboxyl group of 2-oxoglutarate (subsite I) is Lys493 (Figure 5). Mutation of this residue to alanine or histidine inactivated the enzyme completely, but had no inhibitory effect on α<sub>2</sub>β<sub>2</sub> tetramer assembly, whereas mutation to arginine led to ~85% inactivation of the tetramer. The marked increase in the  $K_m$  of the Lys493Arg enzyme for 2-oxoglutarate with no changes in the values for other co-substrates or the peptide substrate indicates that Lys493 is involved in the binding of 2-oxoglutarate. This residue probably constitutes subsite I of the 2-oxoglutarate-binding site, as the  $K_i$  values of the Lys493Arg enzyme for oxoadipinate and pyridine 2,4-dicarboxylate, which become bound at both main subsites of the 2-oxoglutarate site (Majamaa *et al.*, 1984), were distinctly increased, whereas those for oxovalerate and pyridine 2-carboxylate, which cannot bind to subsite I, were unaltered. The  $K_i$  for oxaloacetate, which reacts poorly or not at all at subsite I (Majamaa *et al.*, 1984), was only slightly increased.

The ascorbate-binding site of prolyl 4-hydroxylase also



**Fig. 5.** Critical residues at the  $\text{Fe}^{2+}$ - and 2-oxoglutarate-binding sites of prolyl 4-hydroxylase. The  $\text{Fe}^{2+}$  is coordinated with the enzyme by three residues, His412, Asp414 and His483. The 2-oxoglutarate-binding site can be divided into two main subsites: subsite I consists of Lys493, which ionically binds the C-5 carboxyl group of 2-oxoglutarate, while subsite II consists of two *cis*-positioned equatorial coordination sites of the enzyme-bound  $\text{Fe}^{2+}$  and is chelated by the C-1 carboxyl and C-2 oxo functions (Hanauske-Abel and Günzler, 1982; Majamaa *et al.*, 1984). Molecular oxygen is thought to be bound end-on in an axial position, leading to a superoxide-like structure. His501 is an additional important residue at the catalytic site, probably involved in both coordination of the C-1 carboxyl group of 2-oxoglutarate to  $\text{Fe}^{2+}$  and the decarboxylation of this co-substrate.

contains the two *cis*-positioned coordination sites of the enzyme-bound iron, and is thus partially identical to the binding site of 2-oxoglutarate (Majamaa *et al.*, 1986). Nevertheless, ascorbate does not become bound at subsite I of the 2-oxoglutarate-binding site. In agreement with this, the  $K_m$  of the Lys493Arg enzyme for ascorbate was the same as that of the wild-type enzyme. The  $K_m$  of the Asp414Glu enzyme for ascorbate was likewise not increased, although that for 2-oxoglutarate was increased ~5-fold, again supporting the view that the only main determinant for ascorbate binding is the enzyme-bound iron atom.

His501 appears to be an additional important residue, as its mutation to serine has been found previously to inactivate prolyl 4-hydroxylase by 96% (Lamberg *et al.*, 1995) and its mutation to asparagine or glutamine was now found to inactivate the enzyme by ~95%, while mutation to lysine or arginine inactivated it by 85–90%. This residue was clearly not involved in  $\text{Fe}^{2+}$  binding, however, as the  $K_m$  values of all the His501 mutants for  $\text{Fe}^{2+}$  were the same as those of the wild-type enzyme. In agreement with data on the His501Ser mutant enzyme (Lamberg *et al.*, 1995), the  $K_m$  values of all the His501 mutant enzymes for 2-oxoglutarate were increased ~2- to 3-fold, whereas the  $K_m$  values for ascorbate (Lamberg *et al.*, 1995) and the peptide substrate were not increased significantly. The  $K_i$  of the His501Ser enzyme for pyridine 2,4-dicarboxylate, a competitive inhibitor with respect to 2-oxoglutarate, was also ~2.5-fold (Lamberg *et al.*, 1995). In contrast to the data obtained with the Lys493Arg mutant

enzyme, the  $K_i$  of the His501Ser enzyme for pyridine 2-carboxylate was increased to the same extent as the  $K_i$  for pyridine 2,4-dicarboxylate. These data suggest that His501 may play a role in directing the coordination of the C-1 carboxyl group of 2-oxoglutarate to the enzyme-bound  $\text{Fe}^{2+}$ .

A surprising finding was that the rate of uncoupled decarboxylation of 2-oxoglutarate as a percentage of the rate of the complete reaction was increased up to ~12-fold with all the His501 mutants, but not with any other mutant. This increase in the relative rate of uncoupling was due to a much smaller decrease in the rate of the uncoupled reaction than in that of the complete reaction. This difference cannot be explained by any effect on the binding of the peptide substrate, as the  $K_m$  values of the His501 mutant enzymes for the peptide substrate in the complete reaction and the rate of enhancement by poly(L-proline) in the uncoupled reaction were essentially identical to those observed with the wild-type enzyme. The most likely explanation is that His501 binds through hydrogen bonding to one of the oxygen atoms in the six-membered oxygen-bridged ferryl intermediate (Hanauske-Abel and Günzler, 1982; Hanauske-Abel, 1991) and thus helps the cleavage of the intermediate to succinate,  $\text{CO}_2$  and ferryl ion. As the rate of the uncoupled decarboxylation is only 0.7% of that of the complete reaction, the rate of cleavage of the intermediate may be much more critical in the latter, and thus the His501 mutations can be expected to influence more the rate of the complete reaction. His501 is thus proposed to play two roles in the 2-oxoglutarate-binding site (Figure 5). It directs the orientation of the C-1 carboxyl group of 2-oxoglutarate to the active iron center and accelerates the breakdown of the ferryl intermediate.

## Materials and methods

### Site-directed mutagenesis

Aspartates 391, 414, 419, 422, 442 and 478 (codon GAC or GAT) and lysines 365, 418, 425, 461, 462, 493 and 498 (codon AAA) in the type I  $\alpha$  subunit of human prolyl 4-hydroxylase were converted individually to alanine (codon GCC, GCT or GCA). Asp414 (codon GAC) was converted further to asparagine (codon AAC) and glutamate (codon GAA), and Lys493 to arginine (codon AGA) and histidine (codon ATT). His412 and His483 (codon CAT) were converted to glutamate (codon GAA), and His483 was also converted to arginine (codon CGT). His501 (codon CAT) was converted to arginine (codon CGT), asparagine (codon AAT), glutamine (codon CAA) and lysine (codon AAA). The mutagenesis steps were performed in a pBluescript vector (Stratagene) containing the full-length cDNA clone (PA-59) for the human prolyl 4-hydroxylase  $\alpha$  subunit at the *Sma*I site (Helaakoski *et al.*, 1989). 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 *Afl*III and *Bam*HI. The resulting 1.8 kb *Afl*III–*Bam*HI cDNA fragment containing the mutant site was then cloned into the *Afl*III–*Bam*HI-digested baculovirus transfer vector pVL $\alpha$ 59 (Vuori *et al.*, 1992). The sequences were verified by dideoxynucleotide sequencing (Sanger *et al.*, 1977).

### Generation of recombinant baculoviruses

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 (Gruenwald and Heitz, 1993).

### Analysis of recombinant proteins in insect cells

Sf9 cells were cultured as monolayers in TNM-FH medium (Sigma) supplemented with 10% fetal bovine serum (BioClear) at 27°C. Cells

seeded at a density  $5 \times 10^6$  per 100 mm plate were infected at a multiplicity of 5 with the viruses coding for the wild-type or mutant  $\alpha$  subunit together with a virus coding for the protein disulfide isomerase/ $\beta$  polypeptide (Vuori *et al.*, 1992). The cells were harvested 72 h after infection, washed with a solution of 0.15 M NaCl and 0.02 M phosphate, pH 7.4, homogenized in a 0.1 M NaCl, 0.1 M glycine, 10  $\mu$ M dithiothreitol (DTT), 0.1% Triton X-100 and 0.01 M Tris buffer, pH 7.8, and centrifuged at 10 000 *g* for 20 min. Aliquots of the Triton extracts were analyzed by non-denaturing 8% PAGE and assayed for enzyme activity. The cell pellets were solubilized further in 1% SDS and analyzed by 8% SDS-PAGE under reducing conditions.

### Other assays

Prolyl 4-hydroxylase activity was assayed by a method based on the hydroxylation-coupled decarboxylation of 2-oxo[1- $^{14}$ C]glutarate (Kivirikko and Myllylä, 1982). The reaction was performed in a final volume of 1.0 ml, which contained 10  $\mu$ l of the Triton X-100 extract as the source of the enzyme, 0.1 mg of (Pro-Pro-Gly) $_{10}$ ·9H $_2$ O as substrate, 0.05  $\mu$ mol of FeSO $_4$ , 0.1  $\mu$ mol of 2-oxo[1- $^{14}$ C]glutarate (100 000 d.p.m.), 1  $\mu$ mol of ascorbate, 0.3 mg of catalase (Sigma), 0.1  $\mu$ mol of DTT, 2 mg of bovine serum albumin (Sigma) and 50  $\mu$ mol of Tris-HCl buffer adjusted to pH 7.8 at 25°C. The uncoupled reaction was performed as above, except that the amount of the Triton X-100 extract was increased to 200  $\mu$ l and the peptide substrate was omitted.  $K_m$  values were determined as described previously (Myllylä *et al.*, 1977). Formation of 4-hydroxy-[ $^{14}$ C]proline was studied using [ $^{14}$ C]proline-labeled procollagen substrate (Kivirikko and Myllylä, 1982). Protein concentrations were determined with a Bio-Rad protein assay kit (Bio-Rad). The levels of expression of the wild-type and mutant prolyl 4-hydroxylase tetramers were compared by densitometry of the Coomassie-stained band in non-denaturing PAGE using a BioImage instrument (BioImage, Millipore).

### Acknowledgements

We thank Professor Ari Koskinen, Department of Chemistry, University of Oulu, for valuable comments and suggestions and Eeva Lehtimäki for her expert technical assistance. This work was supported by grants from the Research Council for Health within the Academy of Finland and from FibroGen Inc., Sunnyvale, CA.

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Received on November 5, 1996; revised on December 3, 1996