MISSENSE MUTATIONS THAT CAUSE BRUCK SYNDROME AFFECT ENZYMATIC ACTIVITY, FOLDING AND OLIGOMERIZATION OF LYSYL HYDROXYLASE 2*

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Running head: Biochemical analysis of LH2 with Bruck syndrome mutations

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Bruck syndrome is a rare autosomal recessive connective tissue disorder characterized by fragile bones, joint contractures, scoliosis and osteoporosis. The telopeptides of bone collagen I are underhydroxylated in these patients, leading to abnormal collagen crosslinking. Three point mutations in lysyl hydroxylase 2 (LH2), the enzyme responsible for the hydroxylation of collagen telopeptides, have been identified in Bruck syndrome. As none of them affects the residues known to be critical for LH activity, we studied their consequences at the molecular level by analyzing the folding and catalytic properties of the corresponding mutant recombinant polypeptides. Folding and oligomerization of the R594H and G597V mutants was abnormal and their activity was reduced by more than 95% relative to the wild type. The T604I mutation did not affect the folding properties, although the mutant retained only about 8% activity under standard assay conditions. As the reduced activity was caused by a 10-fold increase in the $K_m$ for 2-oxoglutarate, the mutation interferes with binding of this cosubstrate. In the presence of a saturating 2-oxoglutarate concentration, the activity of the T604I mutant was about 30% of that of the wild type. The T604I mutation did not affect the folding properties, although the mutant retained only about 8% activity under standard assay conditions. As the reduced activity was caused by a 10-fold increase in the $K_m$ for 2-oxoglutarate, the mutation interferes with binding of this cosubstrate. In the presence of a saturating 2-oxoglutarate concentration, the activity of the T604I mutant was about 30% of that of the wild type. However, the T604I mutant did not generate detectable amounts of hydroxylysine in the N telopeptide of a recombinant procollagen I chain when coexpressed in insect cells. The low activity of the mutant LH2 polypeptides is in accordance with the markedly reduced extent of collagen telopeptide hydroxylation in Bruck syndrome, with consequent changes in the crosslinking of collagen fibrils and severe abnormalities in the skeletal structures.

Hydroxylysine residues have at least two important functions in collagens. They are essential for stabilization of the intermolecular collagen crosslinks that provide the tensile strength and mechanical stability for collagen fibrils, and their hydroxy groups serve as attachment sites for carbohydrates, either a galactose or a glucosyl-galactose unit (1-3). Lysyl hydroxylase (LH2, EC1.14.11.4) catalyzes the formation of hydroxylysine in -X-K-G- sequences in a reaction that requires Fe$^{2+}$, 2-oxoglutarate, O$_2$, and ascorbate (1-3). In addition to the -X-K-G-sequence, -X-K-A- or -X-K-S- are hydroxylated in the telopeptides of some fibril-forming collagens, telopeptides being the short nontriple-helical extensions at both ends of collagen molecules. Three vertebrate LH isoenzymes are now known, the overall amino acid sequence identity between the human LH1 and LH2 being 75%, and that between LH3 and the other two isoenzymes 57-59% (4-6). The identity is highest within the C-terminal region that contains the catalytically critical amino acids, two histidines and an aspartate (H638, D640 and H690 in the processed human LH1) that are required for binding Fe$^{2+}$ to the catalytic site, and an arginine (R700 in LH1) that binds the C-5 carboxyl group of 2-oxoglutarate (7, 8). LH2 is expressed in two alternatively spliced forms, long and short, the latter lacking 21 amino acids coded by the 63 bp exon 13A (9, 10). LH2 long is ubiquitously expressed and is the major form in all tissues studied so far and is the only form in skin, lung, aorta and dura, while LH2 short is expressed together with LH2 long in kidney, spleen, liver, cartilage and placenta (9, 10).

The connective tissue disorders Ehlers-Danlos syndrome type VIA (EDS VIA) and Bruck syndrome 2 are caused by mutations in the genes for LH1 (2, 11, 12) and LH2 (13, 14), respectively, and result from abnormal crosslinking of collagens.
EDS VIA is characterized by severe muscle hypotonia, progressive kyphoscoliosis, hypermobile joints, skin fragility, increased risk of fatal arterial ruptures and in some cases ocular fragility (11, 12), while typical features of Bruck syndrome include fragile bones, congenital joint contractures, scoliosis and osteoporosis (13, 14). No mutations in the gene for LH2 were found in patients from one Bruck syndrome family and instead a linkage to chromosome 17p12 was established suggesting that these patients have another defect (13, 15). Patients linked to chromosome 17 are classified as Bruck syndrome 1, whereas patients with LH2 mutations belong to Bruck syndrome 2 (13). This classification is based on genetic data, as no phenotypic differences are observed in the patients (13).

Inactivation of the mouse gene for LH3 leads to embryonic lethality caused by the abnormal synthesis and assembly of collagen IV, resulting in fragmentation of basement membranes (16). LH3 is unique among the LH isoenzymes in that it also possesses collagen glucosyltransferase and galactosyltransferase activities, which reside in its N-terminal domain (17-20). Interestingly, the lethal phenotype of LH3 null embryos is caused by a lack of the glucosyltransferase activity of LH3 and not of its LH activity (21). This glycosyltransferase activity has been shown to be important for the proper assembly and secretion of collagens IV and VI (22) and for cell growth and viability (23). A patient with a human connective tissue disorder caused by mutations in the gene for LH3 was reported very recently to be a compound heterozygote with mutations leading to a marked reduction in the glycosyltransferase and LH activities of LH3 and a decrease in the amount of LH3 polypeptide (24). The patient has several congenital connective tissue malformations that overlap with a number of other collagen disorders (24).

Fibril-forming collagens have four crosslinking sites, one each in the N and C telopeptides and two in the triple helix. Biochemical analyses of tissues from EDS VIA and Bruck syndrome patients have shown that the abnormal collagen crosslinking is caused by underhydroxylation of the crosslinking sites in the triple helix and telopeptides of the fibril-forming collagens, respectively (13-15, 25, 26). Furthermore, increased hydroxylation of collagen telopeptides in various models of fibrosis is correlated with increased levels of LH2 long expression (13, 27, 28). These findings have led to the suggestion that LH1 and LH2 long may be responsible for the hydroxylation of lysine residues within the collagenous and telopeptide regions of collagen polypeptides, respectively. Studies with synthetic peptides, however, have shown that all three LH isoenzymes are capable of hydroxylating the -X-K-G- sequences representing the triple-helical crosslinking sites, but with different efficiencies (29). Analysis of various tissue samples from LH1 null mice recently showed that the triple-helical crosslinking sites were hydroxylated to varying extents in different tissues, thus demonstrating that LH2 and LH3 are also capable of hydroxylating these sites in vivo, although they cannot fully compensate for the lack of LH1 activity (30). The critical role of LH2 long in telopeptide hydroxylation has been confirmed by the recent findings that recombinant LH2 long, but not LH1 or LH3, was able to hydroxylate the -X-K-S- in the N telopeptide of a coexpressed recombinant proα1(I) chain of type I collagen (29) and that no decrease in telopeptide hydroxylation was observed in the LH1 null mice (30).

Three recessive LH2 point mutations leading to the amino acid substitutions R594H, G597V and T604I in LH2 long (Fig. 1) have been identified in Bruck syndrome (13, 14). Both LH2 long and LH2 short transcripts were identified in leukocytes of a control and the R594H proband (14). The relative abundances of the two transcripts in the control and patients samples were presumably similar as the paper did not report on any differences in the splicing ratios (14). LH2 long is a 758-amino-acid polypeptide, the processed LH2 long consisting of 733 amino acids after cleavage of the 25-residue signal peptide. The amino acids of LH2 long are numbered in this paper according to the processed LH2 long polypeptide. The mutations identified in Bruck syndrome are all close to each other but at some distance from the H662, D664 and H714, which correspond to the three conserved Fe2+ binding residues identified in LH1 and several other 2-oxoglutarate dependent dioxygenases, and R724, which corresponds to the conserved basic residue required for binding the C-5 carboxyl group of 2-oxoglutarate (Fig. 1) (7, 8, 31-34). To study the functional and structural consequences of the
R594H, G597V and T604I LH2 long mutations found in Bruck syndrome 2 patients at the molecular level, we expressed recombinant wild-type and mutant LH2 long polypeptides in insect cells, purified them to homogeneity and analyzed their folding and catalytic properties.

**EXPERIMENTAL PROCEDURES**

**Expression of wild-type and mutant recombinant human LH2 long polypeptides in insect cells and their purification** - Generation of the baculovirus transfer vector and the recombinant baculovirus for the expression of a full-length wild-type histidine-tagged human LH2 long with a GP67 secretory signal has been described earlier (18, 35). The mutations R594H, G597V and T604I were introduced into the pAcGP67A-LH2long baculovirus transfer vector using a QuikChange XL Site-Directed Mutagenesis kit (Stratagene). The mutagenesis oligos were the following: R594H, 5’-CTGGGGGAAACATCATGATAGCCATATA TCTGTTGGTTAG-3’ and 5’-CATACACCAGATATGCTATCATGAT GTTTCCTCCCAAG-3’; G597V, 5’-CATAACCAGATATGCTATCATGAT GTTTCCTCCCAAG-3’; G597V, 5’-CATGATAGCCGTATCTTTGTTAGA AATGTTCC-3’ and 5’- GGGACATTTCATAAACAACAGATATACG GCTATCATGATG-3’; T604I, 5’-GGTTATGAAAATGTCCCAATTGATGATTC CACATGAAGC-3’ and 5’-GCTTCATGTGGATATCATCAATTGGGACAT TTTCAATAACC-3’. The DNA sequences were verified on an automated DNA sequencer (ABI Prism 3100, Applied Biosystems). The recombinant baculovirus vectors were cotransfected into *Spodoptera frugiperda* Sf9 cells with BaculoGold DNA (Pharmingen) by calcium phosphate transfection, and the recombinant baculoviruses were amplified (36). The wild-type and mutant LH2 long proteins were expressed in High Five insect cells (Invitrogen) cultured in suspension in Sf900IISFM serum-free medium (Invitrogen). The cells were seeded at a density of 1 x 10⁶ cells/ml and infected at a multiplicity of 5. The medium samples were collected 72 h after infection by centrifugation, first at 250 x g and then at 8,300 x g, for 10 min each. Medium samples containing Complete EDTA-free protease inhibitor cocktail (Roche Applied Science) were incubated with Chelating Sepharose Fast Flow (Amersham Biosciences) resin, 1.5 - 2 ml per 100-ml medium sample, pre-equilibrated with 0.2 M NaCl, 0.1 M glycine, 10 µM dithiothreitol, 1% glycerol, 50 mM urea and 20 mM Tris buffer, pH 7.5, at 4°C overnight with gentle mixing. The resin was packed into a column, the flow-through was collected and the column was washed three times with 10-30 volumes of the above buffer. 300-µl aliquots of the resin with bound proteins were collected before and after washing and boiled directly in SDS-PAGE sample buffer. The bound proteins were eluted from the column with the above buffer containing 50 mM histidine. Samples of the bound proteins before and after washing of the column, and the flow-through, washing and eluted fractions were analyzed by 8% SDS-PAGE under reducing conditions followed by Coomassie Blue staining. Fractions containing the full-length wild-type or mutant LH2 long polypeptides were pooled, concentrated with Amicon Ultra-15 Centrifugal Filter Units (Millipore) and passed through a PD-10 column (Amersham Biosciences) equilibrated with the above buffer to remove the excess histidine, and used directly in LH activity assays. The protein concentrations were determined with RotiQuant (Karl Roth).

**Analysis of wild-type and mutant recombinant human LH2 long polypeptides** - The purified enzymes were analyzed by 6% SDS-PAGE under reducing conditions and 8% non-denaturing PAGE followed by Coomassie Blue staining or ECL Western blotting (GE Healthcare) with a polyclonal rabbit antibody generated against a synthetic LH peptide. N-terminal sequencing was performed using samples transferred to a ProBlott™ membrane (Applied Biosystems) and a Precise™ 492 sequencer (Applied Biosystems) with rapid RP-HPLC gradient. LH activity was determined by a method based on the hydroxylation-coupled decarboxylation of 2-oxo[1-14C]-glutarate in a reaction volume of 0.5 ml using a synthetic peptide (I-K-G), (Innovagen) as a substrate (37). Km values were determined as described earlier (38).

For circular dichroism (CD) spectrum measurements the buffer of the purified wild-type and mutant recombinant LH2 long polypeptides was exchanged to a 10 mM potassium phosphate buffer, pH 7.5, using Amicon Ultra-15 Centrifugal Filter Units (Millipore) according to the manufacturer’s instructions. Circular dichroism
(CD) spectrum measurements of the purified LH2 long polypeptides were recorded with a JASCO J-715 CD spectropolarimeter in a 1-mm path length quartz cuvette, the sample cell temperature being controlled by a JASCO PFD-350S Peltier-type temperature control unit. Far-UV spectra were measured at 20°C between 240 and 195 or 190 nm using a step size of 0.2 nm and a scan speed of 20 nm/min with 4 accumulations for blank spectra and 8 accumulations for sample spectra. The bandwidth used was 1 nm, response time 1 s, and protein concentration 0.3 mg/ml in 10 mM potassium phosphate buffer, pH 7.5.

The purified wild-type and mutant recombinant LH2 polypeptides, 10 µg, were digested with thermolysin (R&D Systems) at a protease:LH2 ratio of 1:500 at 37°C for 60 min. The digestion was stopped by the addition of EDTA to a final concentration of 3 mM. Thermolysin was omitted in control samples. The samples were analyzed by 12% SDS-PAGE under reducing conditions followed by Coomassie Blue staining.

**RESULTS**

**Expression of wild-type and Bruck mutant recombinant LH2 long polypeptides in insect cells and their purification** - The missense mutations R594H, G597V and T604I (Fig. 1) were introduced into the baculovirus transfer vector coding for the long form of LH2 (18) and recombinant baculoviruses were generated. The wild-type and mutant recombinant LH2 long polypeptides containing the GP67 secretory signal and an N-terminal histidine tag (18, 35) were expressed in H5 insect cells and the secreted polypeptides were harvested from the culture medium 72 h after infection and purified by metal chelate affinity chromatography (29). Aliquots of the samples of proteins bound to the column before and after washing and present in the flow-through, wash and elution fractions were analyzed by 8% SDS-PAGE under reducing conditions (Figs. 2 and 3). The wild-type and T604I LH2 long polypeptides became essentially completely bound to the affinity column, while significant amounts of the R594H and G597V mutant polypeptides were found in the flow-through fractions and additional amounts were detached during the washing step (Fig. 2). This may indicate that the histidine tag is partly hidden in the R594H and G597V mutants, while it is readily exposed in the wild-type and T604I LH2 long polypeptides.

Furthermore, the R594H and G597V polypeptides that remained bound after the washing steps were eluted very early after the addition of the elution buffer, in fractions 1-14, together with many additional polypeptides, while the majority of these polypeptides was eluted in fractions 14-30 (Fig. 3).
type and mutant LH2 long polypeptides were pooled, concentrated and passed through a PD-10 column to remove the histidine used in the elution, and the purified enzymes were analyzed by 6% SDS-PAGE under reducing conditions and by 8% nondenaturing PAGE. In contrast to the wild-type and T604I LH2 long polypeptides, a number of additional polypeptides coeluted with the R594H and G597V mutant polypeptides, as evidenced by Coomassie Blue staining (Fig. 4A). Western blot analysis showed that the R594H and G597V mutant polypeptides formed aggregates that were not dissociated into monomer-sized polypeptides in SDS-PAGE (Fig. 4B). In addition, two bands with a lower molecular weight were stained with the LH antibody, indicating that the mutant polypeptides were also prone to degradation (Fig. 4B). N-terminal sequencing showed that the other coeluting bands (Fig. 4A) represented impurities (data not shown).

LH1 isolated from chick embryos or human placental tissues and recombinant LHs 1-3 have been shown by gel filtration to be homodimers (18, 41). Analysis by nondenaturing PAGE showed that the T604I LH2 long polypeptides assembled into dimers as efficiently as the wild-type LH2 long, whereas the oligomerization of the R594H and G597V mutant polypeptides was highly abnormal (Fig. 4). They showed a strong tendency to either aggregate into larger molecular weight complexes that did not enter the gel properly, or to remain as monomers (Fig. 4, C and D). In addition, they formed two kinds of oligomers that had either a faster or a slower mobility than the dimers formed by the wild-type or the T604I dimers (Fig. 4, C and D).

CD spectrum analysis of the wild-type and mutant LH2 long polypeptides - Far-UV CD spectroscopic analysis was performed to study the state of folding of the purified wild-type and mutant recombinant LH2 long polypeptides. The CD spectrum of the wild-type LH2 long is typical of a folded protein and resembles that of a mainly α-helical protein with a positive maximum at 193 nm and negative minima at 208 and 222 nm (Fig. 5). The CD spectrum of the T604I mutant is essentially identical to that of the wild-type LH2 long, suggesting that its folding properties are normal (Fig. 5). By contrast, the R594H and G597V mutants have only one clear negative minimum, at about 207 nm (Fig. 5), indicating dramatic changes in the folding properties of these two polypeptides.

Proteolytic sensitivity of the wild-type and mutant LH2 long polypeptides - The CD spectrum data suggest that the folding of the R594H and G597V mutants is impaired. We analysed this further by subjecting the wild-type and mutant LH2 long polypeptides to limited proteolysis with thermolysin. Thermolysin has been previously shown to digest LH polypeptides in two protease-sensitive regions separating three domains A, B and C (from the N to C terminus) with molecular masses of about 30, 37, and 16 kDa, respectively (18). Thermolysin resistant peptides with similar molecular masses as reported previously (18) were obtained upon digestion of the wild-type and T604I mutant polypeptides, while digestion of the R594H and G597V mutants led to a markedly more extensive proteolysis indicating that these mutant polypeptides are not properly folded (Fig. 6).

Activity and catalytic properties of the wild-type and mutant LH2 long polypeptides - The LH activity of the wild-type and mutant LH2 long enzymes was initially analyzed with an assay based on the hydroxylation-coupled decarboxylation of 2-oxo[1-14C]-glutarate using 800 μM synthetic peptide (I-K-G)₃ as a substrate, 50 μM Fe²⁺, 400 μM 2-oxoglutarate and 1000 μM ascorbate, and 2 μg of the wild-type and mutant LH2 long polypeptides. (I-K-G)₃ was selected as a substrate as LH2 long efficiently hydroxylates this synthetic peptide (29). Typical activity values obtained were about 6000 d.p.m. for the wild-type LH2 long, 500 d.p.m. for the T604I mutant, and < 300 d.p.m. for the R594H and G597V mutants. Thus the reaction velocity of the T604I mutant under these conditions was about 8% of that of the wild type. As differences in the Kₘ values of the wild-type and mutant enzymes for the substrate and/or cosubstrates may affect the observed activities, we determined the Kₘ values. The Kₘ values of the T604I mutant for the peptide substrate, Fe²⁺ and ascorbate were identical to those of the wild type, while the Kₘ for 2-oxoglutarate was about 10-fold relative to that of the wild type (Table 1). In the presence of a saturating 2-oxoglutarate concentration the activity obtained with the T604I mutant was 30% of that of the wild type (data not shown). The Kₘ values of the R594H and G597V mutants for the peptide
substrate were markedly increased, at least 10-fold relative to the wild type (Table 1). Because of the abnormal folding and low activity of these mutants and their extremely high $K_m$ values for the peptide substrate, they were not studied further.

We have shown previously that none of the three recombinant human LH isoenzymes hydroxylates a 23-residue synthetic peptide representing the N telopeptide sequence of the $\alpha_1(I)$ chain of type I collagen (29). However, about 25% of the K9 in the telopeptide of a full-length pro$\alpha_1(I)$ polypeptide chain became hydroxylated by wild-type LH2 long when coexpressed in insect cells indicating that hydroxylation of the telopeptide lysine by LH2 long occurs only in the context of a long peptide (29). We studied next whether the T604I mutant LH2 long is able to hydroxylate the N telopeptide lysine of the full-length pro$\alpha_1(I)$ chain. High Five cells were coinfected with baculoviruses coding for the pro$\alpha_1(I)$ chain, the collagen prolyl 4-hydroxylase $\alpha$ and $\beta$ subunits and wild-type or T604I mutant LH2 long. Control coexpressions were performed without LH2 long. The recombinant procollagen I homotrimers were converted to type I collagen homotrimers by pepsin digestion, purified and analyzed by N-terminal sequencing and amino acid analysis. We have shown previously that pepsin cleaves the N telopeptide of the pro$\alpha_1(I)$ chain between residues G5 and Y6, thus leaving K9 conveniently close to the N terminus for N-terminal sequencing (42). The K9 in the N telopeptide was not hydroxylated when the procollagen chain was coexpressed with the T604I mutant, while hydroxylation of the telopeptide lysine was obtained with the wild-type or T604I mutant LH2 long, respectively, as shown in Fig. 7.

**DISCUSSION**

Collagen fibrils are stabilized by intermolecular crosslinks of two chemically distinct kinds generated by related routes. The allysine route, where crosslinking is initiated by the conversion of a telopeptide lysine to allysine, predominates in the skin, while the hydroxyallysine route, in which a telopeptide hydroxylsine is converted to hydroxyallysine, dominates in stiff connective tissues such as bone, tendon, ligaments and cartilage (15). The pyridinoline crosslinks lysylpyridinolines (LPs) and hydroxylsylpyridinolines (HPs) are derived only via the hydroxyallysine route and can thus be regarded as a measure of telopeptide lysyl hydroxylation (15). The crosslink profile of bone collagen in Bruck syndrome patients is highly abnormal, characterized by an almost complete lack of HP and LP crosslinks and an increased level of allysine-derived crosslinks (15). Three mutations in LH2, which has been identified as the LH isoenzyme responsible for the hydroxylation of telopeptides have so far been reported in Bruck syndrome patients (13, 14, 27-29). As these mutations do not change any of the known catalytically critical amino acids, we studied for the first time their effects on the folding and activity of a recombinant LH2 polypeptide in order to gain an understanding of the molecular pathology of Bruck syndrome.

The three LH2 mutations described in Bruck syndrome patients were introduced into human LH2 long cDNA and the mutant polypeptides were expressed in insect cells and purified. All three mutations led to a marked reduction in LH2 activity. Under standard *in vitro* assay conditions, the activity of the R594H and G597V mutants was < 5% of the wild type. In the case of these two mutations the reduced activity is likely to be primarily due to impaired folding and oligomerization of the mutant polypeptides. LH monomers have been shown to consist of three domains, an N-terminal domain responsible for the glycosyltransferase activity of LH3, a middle domain of unknown function, and a C-terminal domain responsible for LH activity (18). The LH2 long residues R594 and G597 are conserved in LH1 and LH3 and, interestingly, they are located in a region that has been shown to be a protease-sensitive area separating the middle and C-terminal domains in recombinant LH1 and LH3 (18). Furthermore, recombinant C-terminal LH1 and LH3 fragments starting from the amino acid corresponding to K589 in LH2 long have been shown to be fully active LHs (18). It is thus likely that the R594 and G597 residues in LH2 long are part of an unstructured domain boundary, a linker or loop, and that amino acid changes in this region.
may affect the flexibility of this region and the independent folding capacity of the adjacent domains, for instance.

The T604I mutation did not have any apparent effects on the overall folding properties of the LH2 long polypeptide, and the mutant retained about 8% of the wild-type activity under standard in vitro assay conditions. The reduced activity was found to be caused by a 10-fold increase in the $K_m$ for 2-oxoglutarate, while the $K_m$ values for the peptide substrate and the other reaction cosubstrates were identical to those of the wild type. The activity of the T604I mutant at a saturating 2-oxoglutarate concentration was about 30% of that of the wild-type enzyme, but this mutant was not able to generate detectable amounts of N telopeptide hydroxylysine in a recombinant procollagen I chain in cellulo when coexpressed in insect cells. As the $K_m$ of the T604I mutant for 2-oxoglutarate was markedly increased, the mutation clearly affects binding of this cosubstrate. LHs belong to the superfamily of 2-oxoglutarate dioxygenases, the catalytic sites of which are composed of a common 8-stranded $\beta$-helix core fold (jellyroll motif) in which a conserved basic residue in the eighth strand (R724 in LH2) binds the C-5 carboxylate moiety of 2-oxoglutarate (31). This basic residue is located in position +9 or +10 with respect to the second Fe$^{2+}$-binding histidine in all 2-oxoglutarate dioxygenases that have been characterized so far except for the asparaginyl hydroxylase that hydroxylates the hypoxia-inducible factor, where it is located between the Fe$^{2+}$-binding aspartate and the second histidine in the fourth strand (32). In many 2-oxoglutarate oxygenases, additional interactions to the C-5 carboxylate have been shown to be provided by a side chain of a serine or threonine located in position +2 and +4, respectively, relative to the basic residue in the eighth strand (31, 34). Mutation of the corresponding serine in a recombinant human LH1 has been shown to reduce its activity by about 30% although it does not affect the $K_m$ for 2-oxoglutarate (8). Direct hydrogen bonding between the C-5 carboxylate of 2-oxoglutarate or its analogue and the side chains of a threonine in the fourth strand of the jellyroll fold and a tyrosine in the first strand is also common in the enzyme superfamily (31, 34). As the three-dimensional structure of LH is as yet unknown, the exact structural role of the residue T604 in 2-oxoglutarate binding remains to be established.

Taken together, our data show that all three LH2 mutations identified in Bruck syndrome 2 cause a marked reduction in LH2 activity, either by bringing about overall defects in folding and oligomerization of the polypeptide or more specifically by interfering with the binding of one of the necessary cosubstrates, 2-oxoglutarate. None of the three mutations led to complete inactivation of LH2 long, however. This may be important, as our analysis of LH2 null mice has shown that they do not survive beyond embryonic day 12 (unpublished data). These findings suggest that the residual activity of the mutant LH2 long enzymes may be essential for the survival of Bruck syndrome 2 patients. Nevertheless, the low LH2 activity levels lead to markedly reduced extents of hydroxylation of telopeptide lysines, which in turn result in changes in the crosslinking of collagen fibrils and severe abnormalities in the development of skeletal structures.

FOOTNOTES
*This work was supported by the Academy of Finland (grants 1114344 and 1211128) and by the Sigrid Juselius Foundation.

2The abbreviations used are: LH, lysyl hydroxylase; EDS VIA, Ehlers-Danlos syndrome type VIA; CD, circular dichroism; LP, lysylpyridinoline; HP, hydroxylysylpyridinoline

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REFERENCES

**LEGENDS TO FIGURES**

FIGURE 1. Schematic presentation of the locations of the mutations R594H, G597V and T604I reported in Bruck syndrome 2 relative to the catalytically critical residues in the C-terminal part of the processed LH2 long polypeptide. The residues H662, D664 and H714 correspond to the conserved residues known to be critical for binding of the Fe$^{2+}$ atom in LH1 and several other 2-oxoglutarate-dependent dioxygenases, and R724 corresponds to the conserved basic residue required for the binding of the C-5 carboxyl group of 2-oxoglutarate. LH2 long is a 758-amino-acid polypeptide, the processed LH2 long consisting of 733 amino acids after cleavage of the 25-residue signal peptide. The amino acids of LH2 long are numbered according to the processed polypeptide.

FIGURE 2. Analysis of purification of the wild-type and mutant recombinant human LH2 long polypeptides. Wild-type and mutant LH2 long polypeptides were expressed in insect cells and the secreted polypeptides were subjected to purification by metal chelate affinity chromatography. Aliquots of samples of the proteins bound to the column before washing (A), present in the flow-through (B) and wash (C) fractions, and remaining bound to the column after washing (D) were analyzed by 8% SDS-PAGE followed by Coomassie Blue staining. The recombinant LH2 long polypeptides expressed are given above the panels and the position of the wild-type and mutant LH2 long polypeptide is indicated by an arrow.

FIGURE 3. Analysis of elution of the wild-type and mutant recombinant human LH2 long polypeptides from a metal chelate affinity column. The elution fractions were collected and analyzed by 8% SDS-PAGE followed by Coomassie Blue staining. The elution fraction numbers are given above the panels and the positions of the wild-type and mutant LH2 long polypeptides are indicated by arrows.

FIGURE 4. Analysis of the purified wild-type and mutant recombinant human LH2 long polypeptides by SDS-PAGE under reducing conditions and by nondenaturing PAGE. The elution fractions containing the most pure wild-type and mutant LH2 long polypeptides were pooled, concentrated and passed through a
PD-10 column to remove the histidine used in the elution, and the purified enzymes were analyzed by 6% SDS-PAGE under reducing conditions (A and B) and by 8% nondenaturing PAGE (C and D) followed by Coomassie Blue staining (A and C) or ECL Western blotting (B and D). The identity of the polypeptides is given above the panels and the positions of the LH2 long polypeptide (A and B) and LH2 long dimer (C and D) are indicated by an arrow.

FIGURE 5. Far-UV CD spectra of the purified wild-type and mutant recombinant human LH2 long enzymes.

FIGURE 6. SDS-PAGE analysis of the purified wild-type and mutant recombinant human LH2 long polypeptides after thermolysin digestion. Wild-type and mutant LH2 long polypeptides were digested with thermolysin (TL) in a 1:500 protease:LH2 ratio and analyzed by 12% SDS-PAGE under reducing conditions followed by Coomassie Blue staining. The identity of the polypeptides is given above the panel and the position of the undigested LH2 polypeptide is indicated by an arrow.

FIGURE 7. A detail of the elution position of hydroxylysine [Hyl, phenylthiohydantoin (PTH)-Hyl] and lysine (K, PTH-Lys) in the reverse phase HPLC profile of the N telopeptide lysine (K9) of pepsin-digested type I procollagen homotrimers coexpressed with wild-type (A) or T604I mutant LH2 long (B) in Edman degradation sequence analysis. Hydroxylysine gives two major PTH peaks, one that elutes between dimethylphenylthiourea (DMPTU) and PTH alanine and another that elutes between PTH valine and diphenylthiourea (DPTU), the latter being 10-12 times higher than the first one. The first peak was below the detection limit in our analysis. DMPTU and DPTU are byproducts of the sequencing reaction.
**TABLE 1**  
$K_m$ values of the recombinant wild-type and mutant LH2 enzymes for the peptide substrate (I-K-G)$_3$ and the reaction cosubstrate 2-oxoglutarate

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>(I-K-G)$_3$ $K_m$, μM</th>
<th>2-oxoglutarate $K_m$, μM</th>
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<tbody>
<tr>
<td>LH2</td>
<td>400</td>
<td>25</td>
</tr>
<tr>
<td>LH2-R594H</td>
<td>&gt; 5000</td>
<td>n.d.$^a$</td>
</tr>
<tr>
<td>LH2-G597V</td>
<td>&gt; 5000</td>
<td>n.d.$^a$</td>
</tr>
<tr>
<td>LH2-T604I</td>
<td>400</td>
<td>250</td>
</tr>
</tbody>
</table>

$^a$Not determined.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5

\[ \beta_{\text{turn}} \times 10^3 \, (\text{deg cm}^2\text{dmol}^{-1}) \]

\( \lambda \) (nm)

Legend:
- LH2
- LH2-T604I
- LH2-G597V
- LH2-R594H
Figure 6
Figure 7
Missense mutations that cause Bruck syndrome affect enzymatic activity, folding and oligomerization of lysyl hydroxylase 2

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