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Missense Mutations That Cause Bruck Syndrome Affect Enzymatic Activity, Folding, and Oligomerization of Lysyl Hydroxylase 2*

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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 cross-linking. Three point mutations in lysyl hydroxylase (LH) 2, 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 were abnormal, and their activity was reduced by >95% relative to the wild type. The T604I mutation did not affect the folding properties, although the mutant retained only ~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 ~30% of that of the wild type. However, the T604I mutant did not generate detectable amounts of hydroxylysine in the N-terminal 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 cross-linking 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 cross-links 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 (LH[2]; EC 1.14.11.4) catalyzes the formation of hydroxylysine in -*X*KG- sequences in a reaction that requires Fe^{2+}, 2-oxoglutarate, O_2, and ascorbate (1–3). In addition to the -*X*KG-sequence, -*X*KA- or -*X*KS- is hydroxylated in the telopeptides of some fibril-forming collagens, with 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 human LH1 and LH2 is 75%, and that between LH3 and the other two isoenzymes is 57–59% (4–6). The identity is highest within the C-terminal region that contains the catalytically critical amino acids: two histidines and one aspartate (His^{638}, Asp^{640}, and His^{690} in processed human LH1) that are required for binding Fe^{2+} to the catalytic site and one arginine (Arg^{700} 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, with the latter lacking 21 amino acids coded by the 63-bp exon 13A (9, 10). LH2(long) is ubiquitously expressed, is the major form in all tissues studied so far, and is the only form in the skin, lung, aorta, and dura, whereas LH2(short) is expressed together with LH2(long) in the kidney, spleen, liver, cartilage, and placenta (9, 10).

The connective tissue disorders Ehlers-Danlos syndrome type 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 cross-linking of collagens. Ehlers-Danlos syndrome type 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), whereas 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 glycosyltransferase activity of LH3 and not of its LH activity (21). This glycosyltransferase activity has

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[2] The abbreviations used are: LH, lysyl hydroxylase; HPLC, high pressure liquid chromatography.

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 cross-linking sites, one each in the N- and C-terminal telopeptides and two in the triple helix. Biochemical analyses of tissues from Ehlers-Danlos syndrome type VIA and Bruck syndrome patients have shown that the abnormal collagen cross-linking is caused by underhydroxylation of the cross-linking 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 have shown, however, that all three LH isoenzymes are capable of hydroxylating the *-X*KG- sequences representing the triple-helical cross-linking sites but with different efficiencies (29). Analysis of various tissue samples from LH1 null mice showed recently that the triple-helical cross-linking 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 *-X*KS- in the N-terminal telopeptide of a coexpressed recombinant pro-α1(I) chain of type I collagen (29) and that no decrease in telopeptide hydroxylation was observed in LH1 null mice (30).

Three recessive LH2 point mutations leading to the amino acid substitutions R594H, G597V, and T604I in LH2(long) (see 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 patient's samples were presumably similar, as the article did not report on any differences in the splicing ratios (14). LH2(long) is a 758-amino acid polypeptide, with 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 study according to the processed LH2(long) polypeptide. The mutations identified in Bruck syndrome are all close to each other but at some distance from His^{662}, Asp^{664}, and His^{714}, which correspond to the three conserved Fe^{2+}-binding residues identified in LH1 and several other 2-oxoglutarate-dependent dioxygenases, and Arg^{724}, which corresponds to the conserved basic residue required for binding the C-5 carboxyl group of 2-oxoglutarate (see 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 wild-type and mutant recombinant 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 human histidine-tagged full-length wild-type LH2(long) with a gp67 secretory signal has been described (18, 35). The mutations R594H, G597V, and T604I were introduced into the pAcgp67A-LH2(long) baculovirus transfer vector using a QuikChange XL site-directed mutagenesis kit (Stratagene). The oligonucleotides used for mutagenesis were as follows: R594H, 5′-CTGGGGGAAAACATCATGATAGCCATATATCTGGTGGTTATG-3′ and 5′-CATAACCACCAGATATATGGCTATCATGATGTTTTCCCCCAG-3′; G597V, 5′-CATGATAGCCGTATATCTGTTGGTTATGAAAATGTCCC-3′ and 5′-GGGACATTTTCATAACCAACAGATATACGGCTATCATGATG-3′; and T604I, 5′-GGTTATGAAAATGTCCCAATTGATGATATCCACATGAAGC-3′ and 5′-GCTTCATGTGGATATCATCAATTGGGACATTTTCATAACC-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 viruses 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 1×10^6 cells/ml and infected at a multiplicity of infection of 5. The media samples were collected 72 h after infection by centrifugation first at $250 \times g$ and then at $8300 \times g$ for 10 min each. Media samples containing Complete EDTA-free protease inhibitor mixture (Roche Applied Science) were incubated with chelating Sepharose Fast Flow resin (1.5–2 ml/100-ml media sample; Amersham Biosciences) 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 the column and the flow-through, wash, and elution 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 Corp.), 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 Roti-Quant (Karl Roth GmbH).

Analysis of Wild-type and Mutant Recombinant Human LH2(Long) Polypeptides—The purified enzymes were analyzed by 6% SDS-PAGE under reducing conditions and by 8% nondenaturing 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 Procise™ 492 sequencer (Applied Biosystems) with a rapid reverse-phase HPLC gradient. LH activity was determined by a method based on the hydroxylation-coupled decarboxylation of 2-oxo[1-^{14}C]-glutarate in a reaction volume of 0.5 ml using the synthetic peptide $(IKG)_3$ (Innovagen) as a substrate (37). K_m values were determined as described (38).

For CD spectrum measurements, the buffer of the purified wild-type and mutant recombinant LH2(long) polypeptides was exchanged with 10 mM potassium phosphate buffer (pH 7.5) using Amicon Ultra-15 centrifugal filter units according to the manufacturer's instructions. 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, with the sample cell temperature 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 four accumulations for blank spectra and eight accumulations for sample spectra. The bandwidth used was 1 nm; the response time was 1 s; and the protein concentration was 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.

Analysis of Recombinant Collagen Coexpressed with Wild-type and Mutant LH2(Long)—High Five insect cells cultured in suspension were seeded at a density of 1×10^6 cells/ml and co-infected with three recombinant viruses coding for pro-α1(I) chains of type I procollagen (39), the α- and β-subunits of collagen prolyl 4-hydroxylase (a double promoter virus) (40), and wild-type (18) or T604I mutant LH2(long). The pro-α1(I), prolyl 4-hydroxylase, and LH2(long) viruses were used at a ratio of 5:1:1.5–2. L-Ascorbic acid phosphate was added to the culture medium daily to a concentration of 80 μg/ml (40), and the cells were harvested 72 h after infection. The cells were homogenized in 4 volumes of 0.6 M acetic acid, incubated for 2 h, and centrifuged at 10,000 $\times$ *g* for 30 min. The pellet was further dissolved in 2 volumes of 0.6 M acetic acid for 2 h and centrifuged as described above. The supernatants from both centrifugation steps were combined, and NaCl was added to a final concentration of 3 M, followed by the addition of pepsin to a final concentration of 1 mg/ml after a 1-h incubation. The sample was digested for 18 h and centrifuged at 15,000 $\times$ *g* for 30 min. The pellet was washed twice with 2 volumes of 4 M NaCl and 0.05 M Tris buffer (pH 7.4) for 1–4 h and collected by centrifugation as described above. The pellet was dissolved overnight in 2 volumes of 1 M NaCl and 0.05 M Tris buffer (pH 7.4) and centrifuged as described above. Collagen was re-precipitated from the supernatant by the addition of NaCl to a final concentration of 4 M, incubation for 3 h, and centrifugation as described above. The pellet was washed with ⅔ volumes of 4 M NaCl and 0.05 M Tris buffer (pH 7.4) for 1 h and collected by centrifugation at 15,000 $\times$ *g* for 1 h. It was then dissolved in 0.1 M acetic acid, and samples were blotted onto ProBlott™ membrane and subjected to N-terminal protein sequencing with a Procise™ 492 protein sequencer.

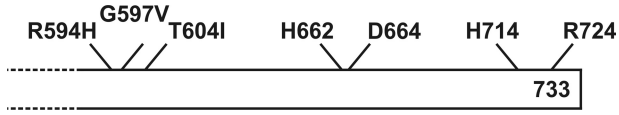

FIGURE 1. **Schematic presentation of the locations of mutations R594H, G597V, and T604I reported in Bruck syndrome 2 relative to the catalytically critical residues in the C-terminal part of the processed LH2(long) polypeptide.** His^{662}, Asp^{664}, and His^{714} correspond to the conserved residues known to be critical for binding of the Fe^{2+} atom in LH1 and several other 2-oxoglutarate-dependent dioxygenases, and Arg^{724} 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; processed LH2(long) consists 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.

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, whereas significant amounts of the R594H and G597V mutant polypeptides were found in the flow-through fractions, and additional amounts were detached during the wash step (Fig. 2). This may indicate that the histidine tag is partly hidden in the R594H and G597V mutants, whereas it is readily exposed in the wild-type and T604I LH2(long) polypeptides. Furthermore, the R594H and G597V polypeptides that remained bound after the wash steps eluted very early after the addition of the elution buffer in fractions 1–14, together with many additional polypeptides, whereas elution of the wild-type and T604I polypeptides started in fractions 12 and 8, respectively, and the majority of these polypeptides eluted in fractions 14–30 (Fig. 3).

The 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

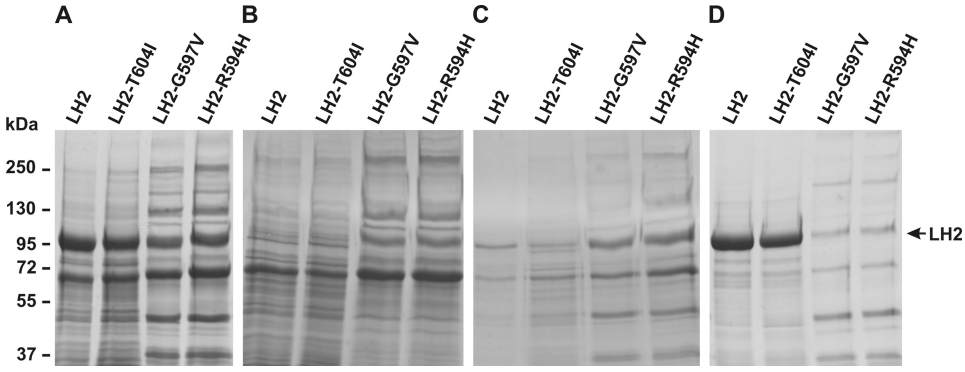

FIGURE 2. **Analysis of purification of the wild-type and mutant recombinant human LH2(long) polypeptides.** Wild-type and mutant LH2(long) polypeptides were expressed in insect cells, and the secreted polypeptides were subjected to purification by metal chelate affinity chromatography. Aliquots of samples 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 indicated above the panels, and the positions of the wild-type and mutant LH2(long) polypeptides are indicated by the *arrow*.

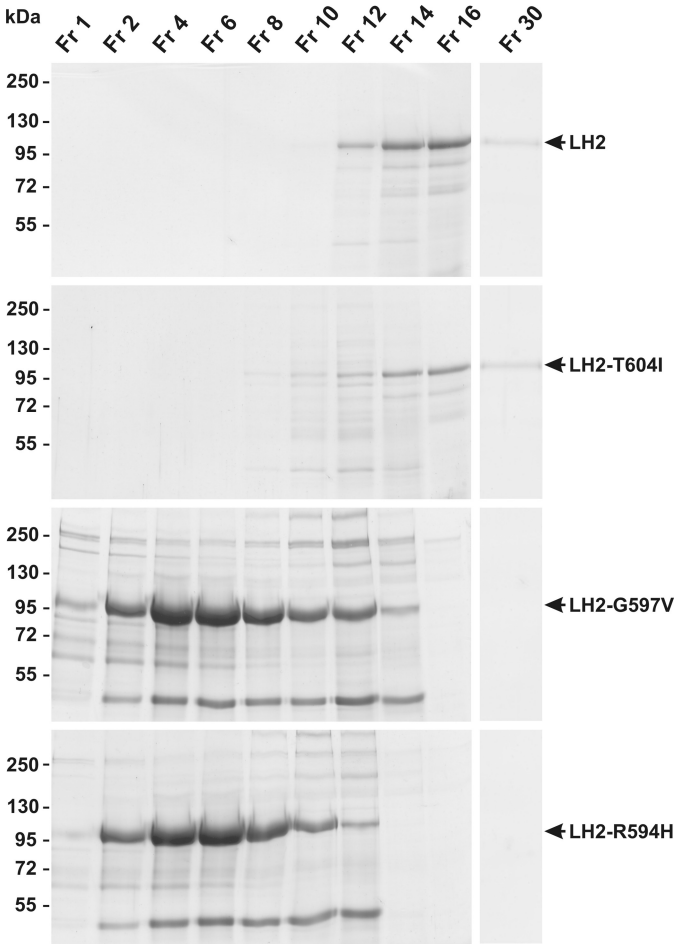

FIGURE 3. **Analysis of elution of the wild-type and mutant recombinant human LH2(long) polypeptides from a metal chelate affinity column.** The elution fractions were collected and analyzed by 8% SDS-PAGE, followed by Coomassie Blue staining. The elution fraction (*Fr*) numbers are indicated above the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

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. 4*A*). Western blot analysis showed that the R594H and G597V mutant polypeptides formed aggregates that were not dissociated into monomer-sized polypeptides upon SDS-PAGE (Fig. 4*B*). In addition, two bands with a lower molecular mass were stained with the anti-LH antibody, indicating that the mutant polypeptides were also prone to degradation (Fig. 4*B*). N-terminal sequencing showed that the other coeluting bands (Fig. 4*A*) represented impurities (data not shown).

LH1 isolated from chick embryos or human placental tissues and recombinant LH1–3 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 wild-type LH2(long), whereas the oligomerization of the R594H and G597V mutant polypeptides was highly abnormal (Fig. 4). They showed a strong tendency either to aggregate into larger molecular mass 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 wild-type or T604I dimers (Fig. 4, *C* and *D*).

CD Spectrum Analysis of 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 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 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 ~207 nm (Fig. 5), indicating dramatic changes in the folding properties of these two polypeptides.

Proteolytic Sensitivity of Wild-type and Mutant LH2(Long) Polypeptides—The CD spectrum data suggest that the folding of the R594H and G597V mutants is impaired. We analyzed this further by subjecting the wild-type and mutant LH2(long) polypeptides to limited proteolysis with thermolysin. Thermolysin has been shown previously 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 ~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, whereas 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).

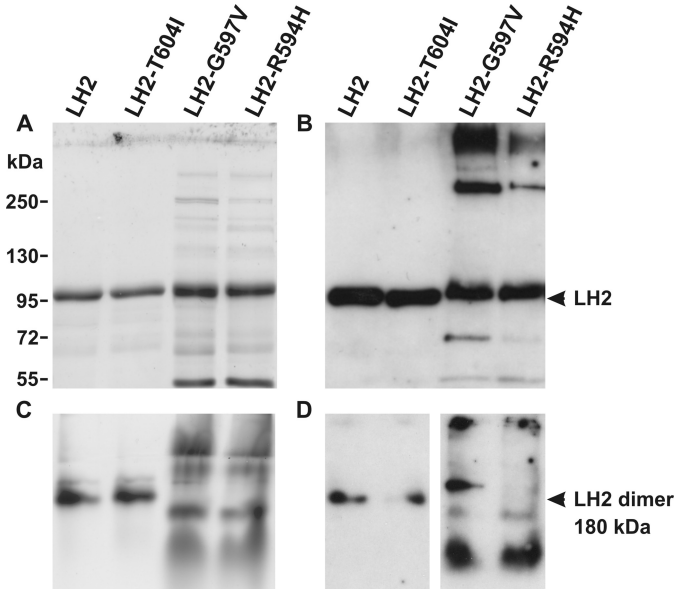

FIGURE 4. **Analysis of the purified wild-type and mutant recombinant human LH2(long) polypeptides by SDS-PAGE under reducing conditions and by nondenaturing PAGE.** The elution fractions containing the most pure wild-type and mutant LH2(long) polypeptides were pooled, concentrated, and passed through 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 indicated above the panels, and the positions of the LH2(long) polypeptide (*A* and *B*) and the LH2(long) dimer (*C* and *D*) are indicated by *arrowheads*.

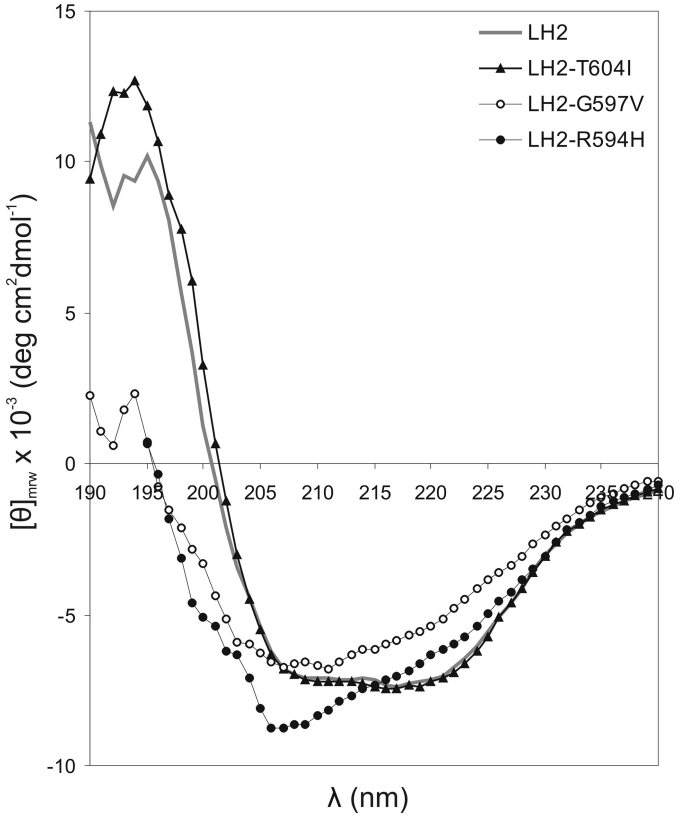

FIGURE 5. **Far-UV CD spectra of the purified wild-type and mutant recombinant human LH2(long) enzymes.** *deg*, degrees.

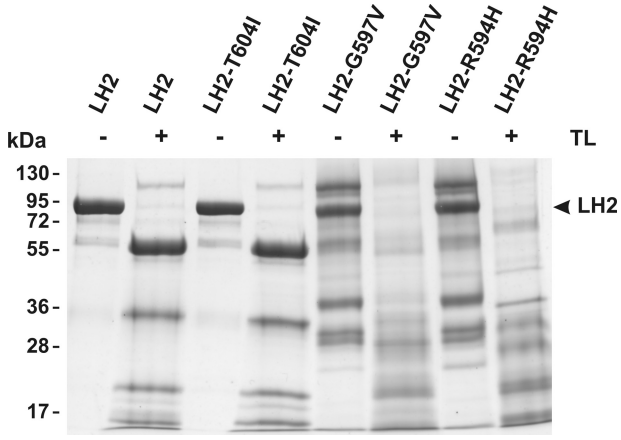

FIGURE 6. **SDS-PAGE analysis of the purified wild-type and mutant recombinant human LH2(long) polypeptides after thermolysin digestion.** Wild-type and mutant LH2(long) polypeptides were digested with thermolysin (*TL*) at a 1:500 protease/LH2 ratio and analyzed by 12% SDS-PAGE under reducing conditions,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

TABLE 1

K_m values of the recombinant wild-type and mutant LH2 enzymes for the peptide substrate $(IKG)_3$ and the reaction cosubstrate 2-oxoglutarate

LH2	K_m for $(IKG)_3$	K_m for 2-oxoglutarate
	μM	μM
Wild type	400	25
R594H	>5000	ND[a]
G597V	>5000	ND
T604I	400	250

[a] Not determined.

Activity and Catalytic Properties of 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-^{14}C]glutarate using 800 μM synthetic peptide $(IKG)_3$ as a substrate, 50 μM Fe^{2+}, 400 μM 2-oxoglutarate, 1000 μM ascorbate, and 2 μg of the wild-type and mutant LH2(long) polypeptides. $(IKG)_3$ was selected as a substrate because LH2(long) efficiently hydroxylates this synthetic peptide (29). Typical activity values obtained were ~6000 dpm for wild-type LH2(long), 500 dpm for the T604I mutant, and <300 dpm for the R594H and G597V mutants. Thus, the reaction velocity of the T604I mutant under these conditions was ~8% of that of the wild type. As differences in the K_m values of the wild-type and mutant enzymes for the substrate and/or cosubstrates may affect the observed activities, we determined the K_m values. The K_m values of the T604I mutant for the peptide substrate, Fe^{2+}, and ascorbate were identical to those of the wild type, whereas the K_m for 2-oxoglutarate was ~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 K_m 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.

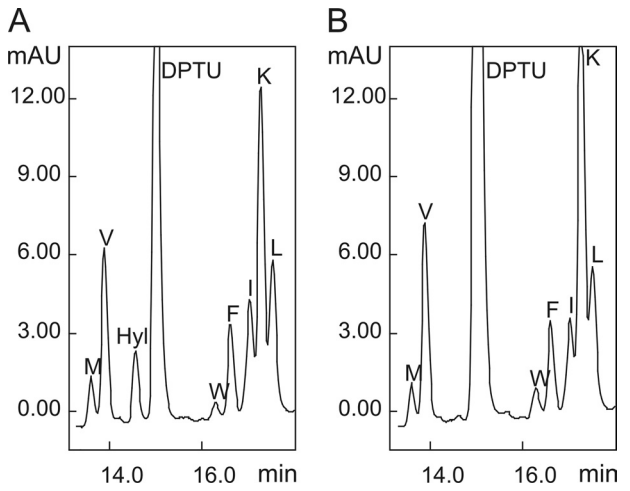

FIGURE 7. **Detail of the elution positions of hydroxylysine (*Hyl,* phenylthiohydantoin-Hyl) and lysine (*K,* phenylthiohydantoin-Lys) in the reverse-phase HPLC profile of the N-terminal telopeptide lysine (Lys^9) of pepsin-digested type I procollagen homotrimers coexpressed with wild-type (*A*) or T604I mutant (*B*) LH2(long) in Edman degradation sequence analysis.** Hydroxylysine gives two major phenylthiohydantoin peaks, one that elutes between dimethylphenylthiourea and phenylthiohydantoin-alanine and another that elutes between phenylthiohydantoin-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. Dimethylphenylthiourea and diphenylthiourea are by-products of the sequencing reaction. *mAU*, milliabsorbance units.

We have shown previously that none of the three recombinant human LH isoenzymes hydroxylates a 23-residue synthetic peptide representing the N-terminal telopeptide sequence of the α1(I) chain of type I collagen (29). However, ~25% of Lys^9 in the telopeptide of the full-length pro-α1(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 T604I mutant LH2(long) is able to hydroxylate the N-terminal telopeptide lysine of the full-length pro-α1(I) chain. High Five cells were co-infected with baculoviruses coding for the pro-α1(I) chain, the collagen prolyl 4-hydroxylase α- and β-subunits, and wild-type or T604I mutant LH2(long). Control coexpressions were performed without LH2(long). The recombinant procollagen I homotrimers were converted to collagen I homotrimers by pepsin digestion, purified, and analyzed by N-terminal sequencing and amino acid analysis. We have shown previously that pepsin cleaves the N-terminal telopeptide of the pro-α1(I) chain between Gly^5 and Tyr^6, thus leaving Lys^9 conveniently close to the N terminus for N-terminal sequencing (42). Lys^9 in the N-terminal telopeptide was not hydroxylated when the procollagen chain was coexpressed with the T604I mutant, whereas hydroxylation of the telopeptide lysine was obtained with wild-type LH2(long) as described previously (29). An example of the elution position of hydroxylysine in the reverse-phase HPLC profile showing the presence and absence of hydroxylysine at Lys^9 when coexpressed with wild-type or T604I mutant LH2(long), respectively, is shown in Fig. 7.

DISCUSSION

Collagen fibrils are stabilized by intermolecular cross-links of two chemically distinct kinds generated by related routes. The allysine route, in which cross-linking is initiated by the conversion of a telopeptide lysine to allysine, predominates in the skin, whereas the hydroxyallysine route, in which a telopeptide hydroxylysine is converted to hydroxyallysine, dominates in stiff connective tissues such as bone, tendon, ligaments, and cartilage (15). The pyridinoline cross-links lysylpyridinolines and hydroxylysylpyridinolines are derived only via the hydroxyallysine route and can thus be regarded as a measure of telopeptide lysyl hydroxylation (15). The cross-link profile of bone collagen in Bruck syndrome patients is highly abnormal, characterized by an almost complete lack of hydroxylysylpyridinoline and lysylpyridinoline cross-links and an increased level of allysine-derived cross-links (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 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 that of the wild type. In the case of these two mutations, the reduced activity is likely to be due primarily 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 Arg^{594} and Gly^{597} 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 Lys^{589} in LH2(long) have been shown to be fully active LHs (18). It is thus likely that Arg^{594} and Gly^{597} 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 ~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, whereas 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 ~30% of that of the wild-type enzyme, but this mutant was not able to generate detectable amounts of the N-terminal 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 β-helix core fold (jellyroll motif) in which a conserved basic residue in the eighth strand (Arg^{724} 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 dioxygenases, additional interactions with the C-5 carboxylate have been shown to be provided by a side chain of a serine or threonine located in position +2 or +4, respectively, relative to the basic residue in the eighth strand (31, 34). Mutation of the corresponding serine in recombinant human LH1 has been shown to reduce its activity by ~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 analog 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 Thr^{604} 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 leads to complete inactivation of LH2(long), however. This may be important because our analysis of LH2 null mice has shown that they do not survive beyond embryonic day 12.[3] 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 cross-linking of collagen fibrils and severe abnormalities in the development of skeletal structures.

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