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# Lysyl-Protocollagen Hydroxylase Deficiency in Fibroblasts from Siblings with Hydroxylysine-Deficient Collagen

**(prolyl-protocollagen hydroxylase/connective tissue/inborn error/crosslinks)**

S. M. KRANE, S. R. PINNELL, AND R. W. ERBE

Departments of Medicine, Dermatology, and Pediatrics, Harvard Medical School and the Medical, Dermatology, and Children's Services, Massachusetts General Hospital, Boston, Massachusetts 02114

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**ABSTRACT** **Cell culture studies were performed on members of a family in which two sisters, ages 9 and 12, have a similar disorder characterized clinically by severe scoliosis, joint laxity and recurrent dislocations, hyperextensible skin, and thin scars. The skin collagen from the sisters was markedly deficient in hydroxylysine, but other amino acids were present in normal amounts. Hydroxylysine in collagen from fascia and bone was reduced to a lesser extent. Since the most likely explanation for the hydroxylysine deficiency was a reduction in enzymatic hydroxylation of lysine residues in protocollagen, we measured the activity of lysyl-protocollagen hydroxylase in crude lysates of cultured skin fibroblasts. Enzyme activities in the two affected children were 14 and 10% of controls, whereas the activity was about 60% of normal in the mother, a pattern most consistent with autosomal recessive inheritance. The mutant enzyme demonstrated the same cofactor requirements as that from normal cells. Deficiency of lysyl-protocollagen hydroxylase is the first inborn error of human collagen metabolism to be defined at the biochemical level.**

Heritable disorders of connective tissue include both dominantly and recessively inherited syndromes (1). Abnormalities in the structural protein, collagen, have been suggested in autosomal, dominantly inherited osteogenesis imperfecta, and in the Marfan and Ehlers–Danlos syndromes (2), but none of the putative biochemical defects has been established as specific for any of these disorders. Autosomal, recessively inherited homocystinuria due to deficiency of cystathionine synthetase shares many of the clinical features of the Marfan syndrome and produces a secondary defect in connective tissue (3). A distinctive mendelian inheritance pattern provides powerful evidence of genetic heterogeneity. We have recently studied a family in which two sisters had some clinical features of the Ehlers–Danlos and Marfan syndromes but in which both parents and an older sister were unaffected, suggesting recessive inheritance (4). The affected 9- and 12-year old sisters had severe progressive scoliosis present since infancy, hyperextensible skin, marked joint laxity and recurrent joint dislocations, and mild arachnodactyly. Ectopia lentis was absent, intelligence was normal, and urinary amino acids were not increased. Amino-acid analysis of dermal collagen from the affected sisters disclosed a marked reduction in hydroxylysine to about 5% of normal, but normal amounts of hydroxyproline and other amino acids. The hydroxylysine content was also reduced in samples of lumbodorsal fascia and variably reduced in bone, but was about normal in a single sample of costal cartilage. The amino-acid compositions of skin from the parents and from the clinically unaffected older sister were normal. The hydroxylysine content of dermis was also normal in three patients, each with the Marfan and Ehlers–Danlos syndromes. Collagen from the skin of the affected children was more soluble in denaturing solvents than that derived from controls (4), consistent with a defect in crosslinking of collagen molecules, a process in which hydroxylysine has been thought to be critically involved (2, 5–8). Hydroxylysine per se is not used in collagen biosynthesis; specific lysyl residues are hydroxylated after their incorporation into the polypeptide chains of protocollagen (9–12). We, therefore, postulated that the basis of this disorder was a deficiency in the enzymatic hydroxylation of lysine. However, lysyl-protocollagen hydroxylase could not be adequately measured either in serum or directly in homogenates of the small amounts of skin available.

In the present investigations, the activity of lysyl-protocollagen hydroxylase measured in lysates of cultured skin fibroblasts from the affected siblings was markedly decreased. Skin fibroblast culture was shown to be a convenient system for further characterization of the mutant enzyme.

## MATERIALS AND METHODS

*Preparation of Fibroblast Lysates.* Fibroblasts from biopsy samples of skin were grown in modified Eagle's medium plus nonessential amino acids (Gibco) containing 15% fetal-calf serum (Gray Industries, Inc.) and harvested by trypsinization 48 hr after reaching confluence. Cells were collected by centrifugation at 1200 × *g*, washed twice by suspension in phosphate-buffered saline (pH 7.2), and collected by centrifugation. The washed cell pellet was suspended at a density of $10^8$ cells per ml in 0.25 M sucrose, 40 mM Tris·HCl (pH 7.4), and 50 mM 2-mercaptoethanol, and the cells were lysed by sonication for 2 min in a Raytheon model DF101 sonic oscillator. The crude lysate was clarified by centrifugation at 50,000 × *g* for 15 min.

Fibroblasts were cultured from the skin of affected and unaffected members of the family with hydroxylysine-deficient collagen (4). Patient 1 refers to the proposita, a 12-year-old girl, and Patient 2 to her affected 9-year-old sister. Cells were also cultured from the 16-year-old unaffected sister, and from the father and mother. Cell cultures from normal controls age-matched to each family member were grown under identical conditions, and lysates were similarly prepared.

*Preparation of Protocollagen Substrates.* Labeled lysyl-protocollagen was prepared by incubation of 50–85 cartilagenous

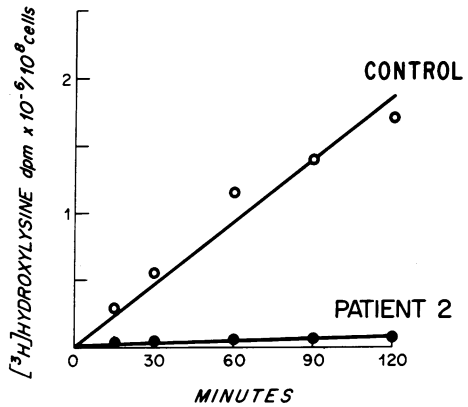


FIG. 1. Lysyl-protocollagen hydroxylase activity in cells of the 9-year-old girl with hydroxylysine-deficiency (Patient 2) compared with age-matched normal control. Formation of [$^3$H]-hydroxylysine is plotted as a function of time and normalized to an equivalent number of cells in the lysates.

tibiae from 10- to 12-day-old chick embryos in 3.0 ml of Krebs "medium A" (13) containing 1 mM $\alpha,\alpha'$-dipyridyl for 30 min at 37° (14, 15). Then, either 125 $\mu$Ci of L-[*U*-$^{14}$C]lysine (250 Ci/mol) or 400 $\mu$Ci of generally labeled L-[$^3$H]lysine (3 Ci/mmol) or 400 $\mu$Ci of L-[*4,5*-$^3$H]lysine (27 Ci/mmol) were added, and the incubation was continued for 2 hr. The tibiae were removed and homogenized at 2°, in 8 ml of distilled water with a Teflon and glass homogenizer, and centrifuged at 100,000 × *g* for 1 hr. The supernatant solution was dialyzed against several changes of 20 mM Tris·HCl (pH 7.6) at 2–4°, then heated in a boiling water bath for 10 min and cooled. The substrate was stored at −20° in 0.5-ml portions. Labeled prolyl-protocollagen was prepared as above with 50 $\mu$Ci of L-[*U*-$^{14}$C]-proline (192 Ci/mol) or 400 $\mu$Ci of L-[*3,4*-$^3$H]proline (4.8 Ci/mmol). Labeled amino acids were purchased from New England Nuclear Corporation.

*Enzyme Assays.* The incubation mixture used for assay of lysyl-protocollagen hydroxylase was similar to that described for prolyl-protocollagen hydroxylase (16) and contained, in a volume of 1.0 ml, lysyl-protocollagen substrate ($^3$H, 0.5–2.0 × 10$^6$ dpm; $^{14}$C, 0.5–2.0 × 10$^5$ dpm), 25 mM Tris·HCl (pH 7.2), 0.1 mM $\alpha$-ketoglutarate, 0.1 mM $FeSO_4$, 0.5 mM ascorbic acid, 0.1 mM dithiothreitol, 2 mg bovine serum albumin, 0.5 mg catalase, and solution of the enzyme to be tested. The reaction was usually initiated by addition of substrate, and samples were incubated at 37° for various periods, usually 90 min. The reaction was terminated by addition of 10 ml of cold (−20°) acetone (15). Reaction mixtures were kept at −20° for at least 30 min and centrifuged at 3000 × *g* for 30 min. The supernatant solution was then aspirated. The pellets were dried in a gentle stream of nitrogen and suspended in 10 ml of 0.3 M citrate–phosphate buffer, pH 6.4. $^{14}$C- or $^3$H-labeled hydroxylysine was measured by oxidizing the terminal carbon to formaldehyde with periodate, coupling with dimedone, and extracting the adduct with 15 ml of toluene by the method of Blumenkrantz and Prockop (17). Radioactivity in 10 ml of the toluene phase was measured with a liquid scintillation spectrometer in counting vials containing 2 ml of 1.5% 2,5-diphenyloxazole and 0.005% *p*-bis[2-(5-phenyloxazolyl)]-benzene in toluene, at an efficiency of about 49% for $^3$H and about 87% for $^{14}$C.

In some experiments we assayed formation of labeled hydroxylysine by stopping the reaction with HCl in a final concentration of 6 N, hydrolyzing for 20 hr at 108°, adding carrier hydroxylysine, drying under reduced pressure, placing the sample on the column of the automatic amino-acid analyzer (18), and collecting the entire effluent in fractions without reacting with ninhydrin. The fractions containing hydroxylysine were identified colorimetrically (19), and aliquots were counted in a thixotropic gel containing 4% Cab-O-Sil (G.L. Cabot, Inc.) in Instagel (Packard Instrument Co.). In other experiments, formation of hydroxylysine was assayed by measurement of the exchange of $^3$H with $H_2O$ after hydroxylation (20). Lysyl-protocollagen substrate labeled with [*4,5*-$^3$H]lysine was used in these assays. The reaction was stopped by addition of trichloroacetic acid in a final concentration of 15%, and the supernatant solution was separated by centrifugation at 3000 × *g*. The supernatant was then distilled under reduced pressure, and 0.5 ml of the distillate was counted in the thixotropic gel.

Most of the assays for lysyl-protocollagen hydroxylase

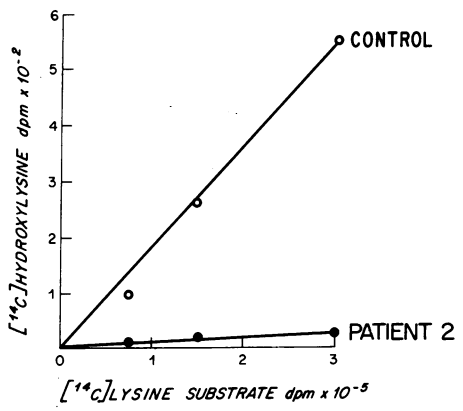


FIG. 2. Lysyl-protocollagen hydroxylase activity in cells of Patient 2 compared with age-matched normal control. Formation of [$^{14}$C]hydroxylysine is plotted as a function of time. Enzyme solution contained the equivalent of 25 × 10$^5$ cells. In this assay, values for [$^{14}$C]hydroxylysine are corrected for recovery of 1 of the 6 labeled carbons. Incubation was for 2 hr at 37°.

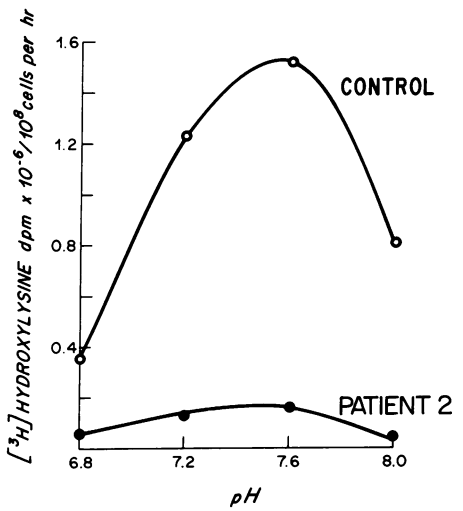


FIG. 3. Lysyl-protocollagen hydroxylase activity in cells of Patient 2 compared with age-matched control, as a function of pH.

were performed with the generally labeled [$^3$H]lysine substrate. Distribution of $^3$H in the lysine molecule is not known. When substrate containing this labeled lysine was incubated with lysyl-protocollagen hydroxylase, and the product was hydrolyzed and chromatographed on the column of the automatic amino-acid analyzer, 66% of the total $^3$H in the hydroxylysine isolated from one batch of the labeled substrate was extractable after oxidation with periodate as [$^3$H]formaldehyde. In the assays to be described, however, results are expressed as dpm of formaldehyde released per $10^8$ cells per hr, and are not corrected for distribution of the label. In any one experiment comparing different cells, the same substrate preparation was used.

Prolyl-protocollagen hydroxylase was assayed by measurement of formation of labeled hydroxyproline (21). Other assays for enzymatic activity in fibroblast lysates included serine hydroxymethyltransferase (EC 2.1.2.1) [modified from (22)] and lactic acid dehydrogenase (EC 1.1.1.27) (23). Protein was measured by the method of Lowry *et al.* with bovine serum albumin as standard (24).

## RESULTS

Activity of lysyl-protocollagen hydroxylase was readily detected in lysates of normal cultured human fibroblasts. Freezing and thawing of the lysates did not affect activity, which was stable for at least 4 months of storage at −20°. With amounts of enzyme equivalent to 1–5 × $10^5$ cells and assaying production of labeled formaldehyde after periodate oxidation, formation of labeled hydroxylysine was linear over a period of incubation of 120 min. The activity in fibroblast lysates from the two affected sisters in the family with hydroxylysine-deficient collagen was about 10% of controls.

In a typical assay, after incubation for 95 min with the equivalent of 5.6 μl of fibroblast lysate from Patient 2, in triplicate, 968, 1,026, and 1,067 cpm were extractable as formaldehyde after oxidation with periodate, whereas with the equivalent of 2.8 μl of lysate from a control, 5,225, 4,781, and 5,444 cpm were extractable, while 88 cpm were extractable from the blank reaction mixture lacking enzyme. Other data for an experiment with cells from Patient 2 are shown in Fig. 1. Because of limited amounts of substrate available, it was necessary to measure activity of lysyl-protocollagen hydroxylase below saturating concentrations for the protocollagen substrates. Nevertheless, the activity of the enzyme in cells from the two affected sisters was deficient over a range of substrate concentrations. Typical results are depicted in Fig. 2. The mean activity compared to age-matched controls was 14 ± 1% in cells from Patient 1 and 10 ± 4% in cells from Patient 2. Activity in all cell lysates was destroyed by heating for 5 min at 60°. The differences in the activity of lysyl-protocollagen hydroxylase in cells from normal and affected individuals were consistent in all of the assays used to measure hydroxylysine formation, *i.e.*, release of labeled formaldehyde from [*U*-$^{14}$C]-lysine or generally labeled [$^3$H]lysine substrates, $^3$H–$H_2O$ exchange from [*4,5*-$^3$H]lysine-labeled substrate, and by column chromatographic isolation of labeled hydroxylysine (Table 1).

TABLE 1. *Different methods of assay of lysyl-protocollagen hydroxylase in fibroblast lysates*

| Assay | Sample | [$^3$H]Hydroxylysine (dpm × $10^{-6}$/$10^8$ cells per hr) | Patient 2 / Control (%) |
|---|---|---|---|
| (*1*) Direct assay | Control | 1.23 | 10.6 |
| | Patient 2 | 0.13 | |
| (*2*) Chromatographic assay | Control | 5.23 | 11.9 |
| | Patient 2 | 0.62 | |
| (*3*) $^3$H–$H_2O$ exchange | Control | 0.689 | 10.9 |
| | Patient 2 | 0.075 | |

Substrates added: Assay *1*, 5 × $10^5$ dpm of generally labeled [$^3$H]lysine substrate; assay *2*, 15 × $10^5$ dpm of generally labeled [$^3$H]lysine substrate; assay *3*, 91 × $10^5$ dpm (*4,5*-$^3$H]lysine substrate.

TABLE 2. *Enzyme assays in fibroblast lysates*

| Enzyme | Activity: Control | Activity: Patient 2 |
|---|---|---|
| Lysyl-protocollagen hydroxylase* (dpm × $10^{-6}$/$10^8$ cells per hr) | 0.778 | 0.090 |
| Prolyl-protocollagen hydroxylase† (dpm × $10^{-6}$/$10^8$ cells per hr) | 0.253 | 0.214 |
| Serine hydroxymethyltransferase μmol/$10^8$ cells per hr) | 0.742 | 0.854 |
| Lactic acid dehydrogenase (μmol/$10^8$ cells per min) | 67.3 | 59.4 |

* [$^3$H]lysyl-protocollagen substrate, 1.4 × $10^6$ dpm.

† [*3,4*-$^3$H]prolyl-protocollagen substrate, 1.4 × $10^6$ dpm. In this assay, labeled hydroxyproline was measured rather than $^3$H–$H_2O$ exchange, accounting for the relatively low activity of prolyl-protocollagen hydroxylase.

The deficiency of lysyl-protocollagen hydroxylase in fibroblasts appeared specific. Activities of three other enzymes tested, prolyl-protocollagen hydroxylase, serine hydroxymethyltransferase, and lactic acid dehydrogenase, were comparable in mutant and control cells as shown for Patient 2 in Table 2. The protein concentration in all cell lysates was similar, averaging 15 mg/ml.

Several characteristics of lysyl-protocollagen hydroxylation were examined in cells from the affected children and compared to normal subjects. The pH optimum for the reaction was about 7.2–7.6 in both normal and mutant cells (Fig. 3); requirements for substrates other than protocollagen were also similar in both (Table 3). The rate of the reaction was reduced markedly by omission of α-ketoglutarate or ascorbic acid and moderately by omission of iron. Further increments in the concentration of α-ketoglutarate to 0.5 mM or greater produced a decrease in rate in both normal and affected cells. The requirement for ascorbic acid was still evident even in the presence of the reducing agent, dithiothreitol. Increasing the concentration of ascorbic acid above 0.5 mM did not increase the rate of lysyl-protocollagen hydroxylation. The need for albumin and catalase for full activity is similar to that described for both lysyl- and prolyl-protocollagen hydroxylases (15, 17, 20, 25) and is as yet unexplained. When the reaction, which was usually done in air, was performed in 100% oxygen, the rate of hydroxylation was actually inhibited to about 50% in all cells tested.

Lysyl-protocollagen hydroxylase was measured in fibroblast lysates from other members of the affected family. As stated

TABLE 3. *Lysyl-protocollagen hydroxylase activity in fibroblast lysates**

| Omission | [$^3$H]Hydroxylysine formed (dpm × $10^{-6}$/$10^8$ cells per hr) | |
|---|---|---|
| | Control | Patient 2 |
| None | 1.64 (1.56–1.78)† | 0.22 (0.20–0.26)† |
| α-Ketoglutarate (0.1 mM) | 0.03 | 0.01 |
| $FeSO_4$ (0.1 mM) | 1.05 | 0.06 |
| Ascorbic acid (0.5 mM) | 0.31 | 0.05 |
| Dithiothreitol (0.1 mM) | 1.75 | 0.23 |
| Bovine serum albumin (2 mg/ml) | 0.51 | 0.02 |
| Catalase (0.5 mg/ml) | 1.14 | 0.14 |

* Complete reaction mixture contained, in addition to components listed above, 25 mM Tris·HCl (pH 7.2) and [$^3$H]lysyl-protocollagen substrate (0.69 × $10^5$ dpm), a different batch from that used in experiments in Table 2. Incubation was for 90 min at 37°.

† $n = 3$.

above, the two sisters with decreased hydroxylysine content of the tissue collagens, on repeated assays, had 14 ± 1 and 10 ± 4% of the activity in control cells. Activity of lysyl-protocollagen hydroxylase in the mother's cells was 60 ± 21% of control. Data on the father's cells and those from the 16-year-old, clinically unaffected sister are at present incomplete.

## DISCUSSION

Hydroxylysine has been assigned a unique role in collagen structure. Either hydroxylysine or its aldehyde, hydroxyallysine, form part of the reducible crosslinks identified in certain collagens (5–8). In addition, hydroxylysine is the site of attachment for carbohydrate in specific residues distributed in collagen polypeptide chains (26, 27).

The deficiency of lysyl-protocollagen hydroxylase observed in skin fibroblasts cultured from two siblings with hydroxylysine-deficient collagen provides a mechanism for the abnormalities observed in this newly recognized clinical syndrome. In normal dermal collagen, hydroxylysine constitutes about 4 residues, and lysine, about 28 per 1000 amino acids. In the affected siblings, collagen hydroxylysine content was reduced to 0.2 and 0.3 residues per 1000, respectively, while the content of lysine was not reduced, consistent with diminished hydroxylation of protocollagen lysyl residues. The resulting aberration in number, type, and distribution of crosslinking in dermal and other collagens (28) could account for the observed physical, chemical, and clinical abnormalities.

A notable feature in the affected siblings is the variation in the degree of hydroxylysine deficit observed in different collagen-containing tissues. While the hydroxylysine content of dermal collagen was reduced to about 5% of normal, collagen from lumbodorsal fascia was reduced to about 20% and bone collagen to about 50% of normal. Among several possible explanations for the tissue-specific variation, regulation of hydroxylysine synthesis by local concentrations of enzyme, substrates, and cofactors is currently under investigation. Optimal concentrations of all components of lysyl-protocollagen hydroxylation did not correct the marked activity deficit in lysates from skin fibroblasts, and the activities were further reduced either by raising or lowering the concentrations of several of the components. Since cofactor requirements for prolyl and lysyl hydroxylation are similar (15, 16, 20, 25), it is unlikely that differences in concentrations of these cofactors would account for consistently normal amounts of hydroxyproline associated with reductions of hydroxylysine to different degrees in different tissues. Comparisons of the substrate, pH, and temperature optima to date have provided no clue as to the nature of the specific molecular lesion in the mutant enzyme.

Other factors might account for the tissue differences, such as multiple forms of lysyl-protocollagen hydroxylase. Whatever the ultimate explanation of the variation in hydroxylysine content of tissue collagens, it seems likely that a widespread, severe deficit would be incompatible with life. In addition to its role in the structure and function of these collagens, hydroxylysine is also an important component of basement membranes (27, 29) and the Clq fraction of hemolytic complement (30).

The observed pattern of lysyl-protocollagen hydroxylase deficiency, with marked reduction in enzyme activity in skin fibroblasts from two siblings and a partial defect in at least one parent, is most consistent with autosomal resessive inheritance, although studies of the father are at present incomplete. It is of interest to note that lysyl-protocollagen hydroxylase activity is readily detected in normal cultured amniotic fluid cells, raising the possibility of prenatal diagnosis of this disorder.

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