Abnormal properties of collagen lysyl hydroxylase from skin fibroblasts of siblings with hydroxylysine-deficient collagen.

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Research Article

Skin fibroblasts from two siblings with hydroxylysine-deficient collagen collagen (Ehlers-Danlos syndrome, type VI) contained normal levels of collagen prolyl hydroxylase activity but were markedly deficient in collagen lysyl hydroxylase activity. The deficiency was evident in all fractions of cell lysates, in low and high ionic strength buffers, and in detergent. Assays of mixtures of wild-type and mutant cell lysates indicated no activation of mutant enzyme by factors in wild-type cells or inhibition of normal enzyme by material in mutant cells. Wild type or mutant cells cultured with ascorbic acid (50 mug/ml of culture medium, added daily) contained approximately the same level of lysyl hydroxylase activity as cells cultured without ascorbate, but prolyl hydroxylase activity without ascorbate was depressed in both an average of 41%. The mutant lysyl hydroxylase was less stable at 37 degrees C than the wild type and did not form high molecular weight aggregates in low ionic strength buffers, as did the control enzyme. The activity of the mutant enzyme was maximally stimulated after dialysis against buffer solutions containing 10 mM dithiothreitol. When assayed in 100 muM dithiothreitol, the mutant enzyme exhibited a higher apparent Km for ascorbate (20 muM) than the wild type (4 muM). In 1.0 mM dithiothreitol the mutant enzyme's apparent Km for ascorbate was reduced to 5 muM. Wild type and mutant enzymes […]

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Abnormal Properties of Collagen Lysyl Hydroxylase from Skin Fibroblasts of Siblings with Hydroxylysine-Deficient Collagen

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A B S T R A C T Skin fibroblasts from two siblings with
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K_m for ascorbate (20 μM) than the wild type (4 μM).
In 1.0 mM dithiothreitol the mutant enzyme's apparent
K_m for ascorbate was reduced to 5 μM. Wild type and
mutant enzymes had the same apparent K_m for α-keto-
glutarate (20 μM). The properties of prolyl hydroxylase
in wild type and mutant cells were identical: apparent
K_m's for ascorbate and α-ketoglutarate were 100 μM and
20 μM, respectively. If mutant enzyme protein with al-
tered kinetic properties is the only enzyme functioning
to hydroxylate lysyl residues in collagen, the variations
in hydroxylysine content observed in collagen from dif-
ferent tissues in the subjects reported here could be in
part due to differences in cofactor concentrations and in
rate and sequence of events in collagen synthesis in dif-
ferent tissues.

INTRODUCTION

Current knowledge of the events in collagen metabolism
is such that the biochemical basis of certain connective
tissue disorders can now be explained. Human hydroxy-
lysine-deficient collagen disease (Ehlers-Danlos syn-
drome, type VI) is a disorder characterized by a spe-
cific aberration in collagen synthesis. This heritable dis-
ease was first identified in two sisters with features sug-
gestive of both Ehlers-Danlos and Marfan syndromes
(1). The clinical manifestations of the disease included
kyphoscoliosis, joint laxity with recurrent joint disloca-
tions, hyperextensible skin, and slow wound healing.
Amino acid analysis of the dermis from the two children
showed that the skin collagen contained a normal amount
of hydroxyproline, but the hydroxylysine content was
markedly reduced to approximately 5% of normal. The
hydroxylysine content of other tissues was reduced to a
variable extent; fascia contained 14–28%, bone about
43%, and cartilage 90% of the amount present in corre-
sponding tissues from normal subjects. The skin collagen
from these patients was abnormally soluble in denaturing
solvents such as 4 M CaCl₂ and 9 M KSCN. These
observations were consistent with a deficiency of hy-
droxylysine-derived intermolecular cross-links, and
analysis of borohydride-reducible cross-links indicated
abnormalities in the nature, distribution, and amount of
these moieties in the dermal collagen (2).

Hydroxyproline and hydroxylysine are formed by en-
zymatic oxidation of prolyl and lysyl residues in pre-
cursor component chains of the collagen molecule. The
hydroxylations are catalyzed by two separate enzymes,
prolyl hydroxylase, (prolyl-protocollagen hydroxylase

or collagen prolyl hydroxylase EC 1.14.11.2), and lysyl hydroxylase (lysyl-protocollagen hydroxylase or collagen lysyl hydroxylase). Lysates of cultured skin fibroblasts from the affected siblings contained normal levels of prolyl hydroxylase activity but reduced lysyl hydroxylase activity (less than 15% of that present in fibroblasts from normal subjects) (3). In vitro assays of prolyl and lysyl hydroxylase activity in crude lysates of normal and mutant cells require the presence of oxygen, α-ketoglutarate, ascorbic acid, and ferrous ion for the hydroxylation reactions to occur at optimal rates. Thus the enzymes in human skin fibroblasts have characteristics in common with prolyl hydroxylases from human and rat liver (4, 5), newborn rat skin (6), chick embryos (7), and mouse L-929 fibroblasts (8, 9), as well as lysyl hydroxylases from chick embryo (10, 11) and fetal rat skin (12).

Further studies of collagen hydroxylases in skin fibroblasts were performed to learn more about human enzymes from this source. Comparison of the properties of hydroxylases from normal and deficient cells might thus help to elucidate the cause of defective collagen lysyl hydroxylation. This communication reports studies of characteristics of collagen lysyl and prolyl hydroxylases in normal cells and in cells from two siblings with hydroxylysine-deficient collagen (3). The lysyl hydroxylase from deficient cells has chemical, physical, and kinetic properties significantly different from the enzyme in normal cells.

METHODS

Fibroblast lysates. Cells were grown in 100-mm diameter plastic petri dishes (Falcon Plastics, Division of BioQuest, Oxnard, Calif.) or in glass roller bottles, the latter with an approximate growth surface area of 600 cm³. Mutant 1 and 2 refer to cells grown from patients 1 and 2, respectively, with Ehlers-Danlos syndrome type VI (1, 3). Most cultures were in passage 10–20. Control cells used in earlier studies were from normal subjects age-matched for the mutant subjects. Some of these cells were no longer available during the present studies. All control cells used in the present experiments were from children aged 3 wk–18 yr and matched approximately for passage number and growth rate. The culture medium, modified Eagle's medium (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 15% fetal calf serum, glutamine, essential and nonessential amino acids, glucose (1.5 mg/ml), sodium pyruvate (0.22 mg/ml), and $Fe(NO_3)_3 \cdot 9$ H_2O (1 mg/ml) was changed twice weekly. Cultures 2 days after confluence were harvested by trypsinization, cells were counted in a hemocytometer, separated from the medium by centrifugation at 1,200 g for 10 min, washed three times with phosphate-buffered saline, and then suspended at a density of 0.1–1 × 10⁸ cells/ml in 40 mM Tris-HCl (pH 7.4), 250 mM sucrose, and 50 mM 2-mercaptoethanol (2-ME).[1] All operations after trypsinization were done at 4°C. The cell suspension was lysed by sonication for 2 min in a Raytheon model DF 101 sonic oscillator (Raytheon Co., Sorensen Operation, Production Equipment Dept., Norwalk, Conn.). The lysate was centrifuged at 50,000 g for 60 min, and the supernate was carefully separated from the overlying lipid layer. In some experiments acetone powders were prepared according to the method of Morton (13).

Protocollagen substrate. The method of preparation of [3,4-³H]proline or [G-³H]lysine (New England Nuclear, Boston, Mass.) protocollagen substrate from chick embryo tibia (4, 11) as modified by Krane et al. (3) was used. Although the same method of substrate preparation was always followed, for unexplained reasons different substrate preparations exhibited variable activities with the same enzyme preparation.

With constant amounts of control human fibroblast lysate and incubation times of 60–120 min, a plateau in hydroxylysine formed was not reached with amounts of underhydroxylated collagen substrates tested up to 10 times those used in most assays reported here. Because of the amounts of radioactivity necessary and the relatively low yields of underhydroxylated substrate, it was not practical to determine saturating conditions for every substrate preparation or to attempt to employ saturating collagen substrate conditions in every assay.

Enzyme assays. Assays for lysyl or prolyl hydroxylase contained (in a total volume of 1.0 ml) 25 mM Tris-HCl (pH 7.2), 100 μM α-ketoglutarate (Sigma Chemical Co., Inc., St. Louis, Mo.), 100 μM ferrous sulfate, 500 μM ascorbic acid (recrystallized, Fisher Scientific Co., Pittsburgh, Penn.), 0.5 mg catalase (Sigma), 100 μM dithiothreitol (DTT) (Calbiochem, San Diego, Calif.), 2 mg of bovine serum albumin (Miles Laboratories, Inc., Elkhart, Ind.) cell lysate (usually 10–25 μl of lysates from sonicated cells suspended at 0.1–1 × 10⁸ cells/ml) and 0.5–2.5 × 10⁵ dpm of protocollagen substrate. The enzyme reactions were initiated by addition of either substrate or enzyme and, unless otherwise indicated, assay mixtures were incubated for 60–120 min in air at 37°C. Prolyl hydroxylase assays were terminated by the addition of 0.5 ml of 15% trichloroacetic acid. Tritiated water produced on hydroxylation of labeled proline was separated from the reaction mixture by vacuum distillation. Portions of the distillate were counted in 10 ml of Insta-gel (Packard Instrument Co., Inc., Downers Grove, Ill.) containing 3 ml of H_2O with an efficiency of approximately 30%. Lysyl hydroxylase assays were terminated by addition of 10 ml of cold ($-20°C$) acetone. The labeled formaldehyde produced by periodate oxidation of carbon 6 of [G-³H]hydroxylysine was coupled with dimedone, and the complex was extracted into 15–20 ml of toluene (3, 14). 10 ml of the toluene phase was added to a scintillation counting vial containing 2 ml of 1.5% 2,5 diphenyloxazole and 0.005% p-bis [2-(5-phenyloxazolyl)]-benzene in toluene. Radioactivity was assayed with a Packard model 3375 Tricarb scintillation spectrometer.

Lysyl hydroxylase activities were usually expressed as disintegrations per minute of [³H]formaldehyde produced per 10⁸ cells per hour, and prolyl hydroxylase activities were expressed as distintegrations per minute [³H] water formed per 10⁸ cells per hour. The absolute values of enzyme activities can only be compared when identical substrate preparations are used. In any one experiment, the same substrate preparation (prolyl or lysyl) was utilized in all assays. Lactate dehydrogenase activity was measured by the method of Klevecz and Ruddle (15).

Measurements of apparent K_m for cosubstrates. Under standard assay conditions, the rate of formation of [³H]-

hydroxylysine by enzymes in wild-type and mutant cells was linear for at least 60 min. Apparent K_m values were measured by incubation for 30–40 min with variation of the concentration of one substrate.

Ascorbate concentrations. Ascorbate concentrations were determined by modified spectrophotometric methods utilizing diazotized 4 methoxy-2-nitroaniline (16, 17). Standard ascorbate solutions of 5–50 μg/ml (in 25 mM Tris-HCl, pH 7.2) were prepared by dilution of a stock solution containing 500 μg/ml. Color reagent was prepared by dissolving 500 mg of 4-methoxy-2-nitroaniline in glacial acetic acid (125 ml) and diluting the solution to 250 ml with 10% aqueous sulfuric acid. The color reagent (2 ml) was diazotized by reaction with 2 ml of fresh 0.2% sodium nitrite solution, and 75 ml of absolute ethanol was added as soon as the reaction mixture became colorless. Then 1.0 ml of diazotized color reagent solution was immediately added to 1.0 ml of ascorbate solutions and mixed for 1 min, and 0.5 ml of 10% sodium hydroxide was added. The blue product exhibited an absorption maximum at 570 nm. A standard curve relating absorbance at 570 nm versus ascorbate concentration was linear in the range 5–100 μg/ml. Ascorbate concentrations in freshly prepared solutions of dehydroascorbic acid (in 25 mM Tris-HCl, pH 7.2) containing varying concentrations of DTT were established by comparison with the standard curve.

RESULTS

Collagen hydroxylase activities in normal and deficient cells

Whole cell lysates of mutant cells and 50,000 g supernates contained levels of prolyl hydroxylase activity within the range of that in control cells, but in both preparations the lysyl hydroxylase activity in mutant cells was markedly lower than that in any control cell (Table I). Because cells from different age-matched normal subjects cultured under identical conditions contain variable levels of lysyl hydroxylase, prolyl hydroxylase, and lactate dehydrogenase activities, exact quantitation of a deficiency of lysyl hydroxylase activity can be arbitrary. In any given cell lysate, the ratio of the activity of collagen lysyl hydroxylase to that of collagen prolyl hydroxylase may best reflect relative lysyl hydroxylase activity, since both enzymes are presumed to be involved only in the biosynthesis of collagen.

Sonication of cell suspensions in buffer containing 0.1% Triton X-100 did not significantly increase the collagen lysyl hydroxylase activity present in the 50,000 g supernate of normal or mutant cell lysates. Suspensions of the 50,000 g pellets (in 40 mM Tris-HCl, pH 7.4, 250 mM sucrose, and 50 mM 2-ME) and extracts of acetone powders prepared from such suspensions contained residual lysyl and prolyl collagen hydroxylase activities, but all such preparations from mutant cells were deficient in lysyl hydroxylase activity. Though collagen lysyl hydroxylase from chick embryos has been found to be relatively insoluble in low ionic strength buffers (11), we found similar lysyl hydroxylase activity in 50,000 g supernates of fibroblast lysates in both high

and low ionic strength buffer (20 mM Tris-HCl buffer [pH 7.4], 200 mM NaCl, 100 mM glycine, and 50 μM DTT or 40 mM Tris-HCl [pH 7.4], 250 mM sucrose, and 50 mM 2-ME). These studies also indicated that the presence of 50 mM 2-ME in the extracting buffer had no observable effect on either prolyl or lysyl hydroxylase activities.

There was no inhibition of the activity of lysyl hydroxylase from normal cells by proportionate amounts of mutant cell lysate, as shown in Fig. 1. To date we have not observed any change in the activities of collagen hydroxylases in control cells maintained in culture with repeated passages. However, we have recently found that certain cultured late-passage mutant cells contained a level of lysyl hydroxylase activity 25–35% of the activity in a given control cell (versus 2–15% in early-passage cells), although the level of prolyl hydroxylase activity was unchanged.
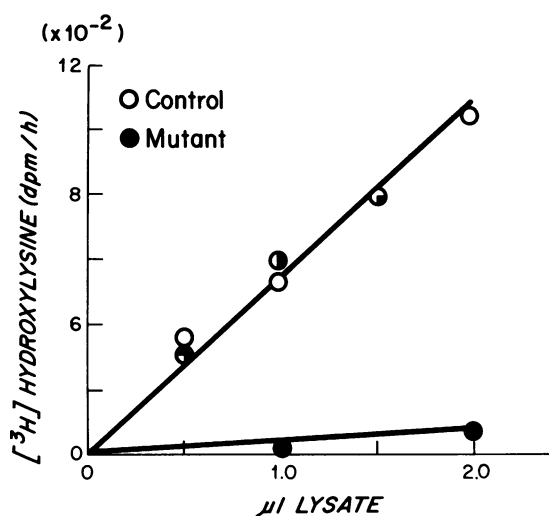
(x10⁻²)

FIGURE 1 Lysyl hydroxylase activity in mixtures of wild-type and mutant fibroblast lysates. The 50,000 g supernates of normal or mutant cell lysates (10⁸ cells/ml) were diluted 1:19 with 40 mM Tris-HCl (pH 7.4), 250 mM sucrose, 50 mM 2-ME before assay. Mixtures assayed contained the amount of normal control lysate indicated by the abscissa plus a proportionate amount of mutant cell lysate indicated by the shaded area.

Ascorbate and enzyme levels

Lysyl hydroxylase activity in multiplying fibroblast cultures roughly paralleled the number of cells in the culture (Fig. 2A). At all time points examined after planting of the culture, the deficiency of lysyl hydroxylase activity in the mutant cells was evident. Daily supplementation of the culture medium with ascorbate (to 50 μg/ml) produced no significant change (− 15– + 25%) in lysyl hydroxylase activity in cultures of normal or mutant cells, although in one of three experiments the activity in mutant cells rose eightfold (but only to a level 12% of control).

Prolyl hydroxylase activity in wild type and mutant cells was affected adversely by the continuous presence of ascorbate in the culture medium. In three experiments (one of which is shown in Fig. 2B), at every time point studied, prolyl hydroxylase activity in cultures supplemented daily with ascorbate was less than the activity in unsupplemented cultures. At confluence, cultures of mutant and normal cells maintained in ascorbate contained from 19–61% (average .41%) of the prolyl hydroxylase activity present in unsupplemented replicate cultures.

Sulfhydryl compounds and enzyme activity

Lysyl and prolyl hydroxylase activities were usually measured in assay mixtures containing 100 μM DTT, as suggested by Rhoads and Udenfriend (6). We found

that omission of DTT at this concentration had little effect on the rate of lysyl or prolyl hydroxylation by enzymes in cell lysates prepared and stored in buffer containing 50 mM 2-ME; however, cell lysates dialyzed against buffers lacking 2-ME required DTT in the assay solution for significant rates of prolyl and lysyl hydroxylation (Table II). Para-chloromercuribenzoate (PCMB) inhibited both normal and mutant lysyl hydroxylases when preincubated with lysates in buffer lacking 2-ME but containing cofactors (ascorbate,
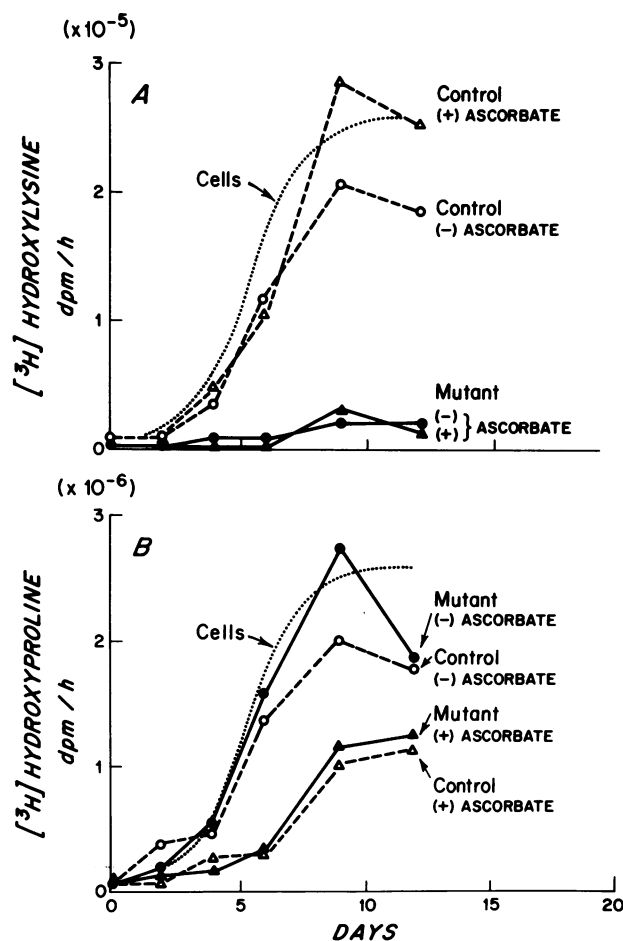
FIGURE 2 Collagen lysyl and prolyl hydroxylase activities in cell cultures grown with and without added ascorbate in the culture medium. On day 0, replicate cultures were seeded with 2 × 10⁵ cells in 100-mm diameter plastic petri dishes (Falcon). Ascorbate (final concentration 50 μg/ml) was added daily (from day 0) to one half of the cultures. Culture medium was changed twice weekly. (●) Enzyme activity in mutant cells cultured without ascorbate. (▲) Activity in mutant cells cultured with ascorbate. (○) Activity in wild-type cells cultured without ascorbate. (△) Activity in wild-type cells cultured with ascorbate. (· · · ·) cells/five plates (at confluence approximately 25 × 10⁶ cells) (For purposes of illustration the mean number of cells for all cultures is shown).

TABLE II
Effect of Sulfhydryl Compounds and PCMB on Collagen Hydroxylase Activities

Cells	2-ME in buffer	[DTT] in assay	PCMB	Activity	
				Lysyl hydroxylase	Prolyl hydroxylase
	mM	mM		dpm × 10⁻⁶/10⁸ cells/h	
Mutant 2	0	0	0	0.06	1.88
	0	0.1	0	0.13	15.9
	0	0.1	+	0.07	N.D.
	50	0	0	0.24	9.90
	50	0.1	0	0.29	11.5
Control 1	0	0	0	0.21	0.38
	0	0.1	0	4.47	14.8
	0	0.1	+	0.14	N.D.
	50	0	0	4.29	10.1
	50	0.1	0	4.65	9.7

Cell lysates were diluted 10-fold with 40 mM Tris-HCl (pH 7.4), 250 mM sucrose with or without 50 mM 2-ME, and dialyzed at 4°C for 72 h against the same buffer. In some experiments PCMB (final concentration, 0.1 mM) was added to enzyme in assay solution containing cofactors but not DTT or protocollagen substrate. The assay was initiated by addition of the latter two ingredients immediately after PCMB was added.

α-ketoglutarate, and ferrous sulfate). The inhibition by PCMB was still apparent even though DTT (to 100 μM) was added at the time of assay.

When fibroblast lysates were dialyzed for 24 h at 4°C against buffer containing a relatively high concentration of DTT (10 mM), the activities of prolyl hydroxylases from normal or mutant cells and the lysyl hydroxylase from normal cells were usually unaffected, but the activity of lysyl hydroxylase from mutant cells was invariably significantly enhanced (Table III). In other experiments performed under the same conditions, the activity of the mutant enzyme was less dramatically stimulated (twofold increase in activity compared to fourfold observed in some experiments). After prolonged dialysis versus buffers containing 10 mM DTT (48 h or more at 4°C) both normal and mutant lysyl hydroxylases lost considerable activity (50% or more).

When lysates containing 10 mM DTT were added to the assay mixture, the resultant DTT concentration was higher than 100 μM. If the dialysis step was eliminated and the DTT concentration in the assay mixture was increased from 0.1 to 1 mM, the rate of collagen hydroxylation by the mutant lysyl hydroxylase was increased while prolyl hydroxylase activity was either slightly increased or decreased. The activity of the mutant enzyme was always higher when assayed in 1 mM DTT, though the stimulation of activity often varied between experiments (+ 20–+ 200%). The activity of normal lysyl hydroxylase was usually unaffected or slightly enhanced by 1 mM DTT, although occasionally

as much as 50% loss in activity was noted (Table IV). Studies of enzyme activity in varying concentrations of DTT (to 10 mM) indicated that the mutant lysyl hy-

TABLE III
Lysyl Hydroxylase Activities after Dialysis

Cells	Buffer	[DTT] in assay	Activity lysyl hydroxylase
		mM	dpm × 10⁻⁶/ 10⁸ cells/h
Mutant 1	50 mM Tris-HCl (pH 7.4) 10% glycerol	0.1	1.83
	50 mM Tris-HCl 150 mM NaCl	0.1	1.68
	50 mM Tris-HCl 10% Glycerol 10 mM DTT	2.0	6.40
	50 mM Tris-HCl 150 mM NaCl 10 mM DTT	2.0	5.07
Control	50 mM Tris-HCl 10% glycerol	0.1	7.49
	50 mM Tris-HCl 150 mM NaCl	0.1	8.09
	50 mM Tris-HCl 10% glycerol 10 mM DTT	2.0	7.07
	50 mM Tris-HCl 150 mM NaCl 10 mM DTT	2.0	6.74

Lysates from 10⁷ cells were diluted 10-fold in the indicated buffers and then dialyzed overnight against the same buffer, and 0.2 ml of the dialyzed enzyme solution was assayed.

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TABLE IV

Ascorbate and DTT and Activity of Hydroxylases

Cells	[DTT]	[Ascorbate]	Hydroxylase activity	
			Lysyl	Prolyl
	mM	mM	$dpm \times 10^{-5}/$ 10^8 cells	$dpm \times 10^{-6}/$ 10^8 cells
Experiment I				
Mutant 1	0.1	0.5	0.27	7.12
	0.1	0	0.03	0.57
	1	0.5	0.39	8.59
	1	0	0.16	3.58
	5	0.5	0.31	7.07
	5	0	0.28	5.81
	10	0.5	0.26	5.33
	10	0	0.19	4.39
Control	0.1	0.5	1.65	8.24
	0.1	0	0.17	1.43
	1	0.5	0.71	10.4
	1	0	0.30	3.41
	5	0.5	0.50	7.55
	5	0	0.41	5.57
	10	0.5	0.27	5.35
	10	0	0.24	5.05
Experiment II				
Mutant 2	0.1	0.5	0.58	2.83
	1.0	0.5	1.35	3.13
Control	0.1	0.5	1.56	2.89
	1.0	0.5	1.85	3.22

droxylase was most active in the presence of 500 μM ascorbate and 1 mM DTT. Prolyl and lysyl hydroxylases from normal and mutant cells exhibited little or no activity when assayed in 0.1 mM DTT without added ascorbate. However, with 1–10 mM DTT these enzymes could function in the absence of ascorbate (though suboptimally), presumably by utilizing DTT as an alternative reducing agent.

In an effort to determine if DTT-induced stimulation of mutant lysyl hydroxylase activity could be achieved in vivo, cell cultures were incubated in medium containing 10 mM DTT for 1 h. This treatment did not increase lysyl hydroxylase activity; rather prolyl and lysyl hydroxylase activities in normal and mutant cells were decreased 30–35%.

Cofactor and cosubstrate requirements

Ascorbate. Measurements of the apparent K_m for ascorbate revealed a discernible difference between wild-type and mutant lysyl hydroxylases (Fig. 3A and C). The enzyme in mutant cells had an apparent K_m for this cofactor (20 μM) approximately five times that of the normal enzyme (4 μM). The ascorbate concentration routinely present in assay mixtures (0.5 mM) was approximately 25 times the apparent K_m of the mutant enzyme. Higher ascorbate concentrations (to 2.5 mM) did not increase enzyme activity. The DTT concentration affected the

apparent K_m of mutant enzyme for ascorbate. In the presence of 1 mM DTT, the mutant enzyme exhibited a greater affinity for ascorbate (apparent K_m 5 μM) (Fig. 3B), while the apparent K_m of the normal enzyme was the same in 0.1 or 1.0 mM DTT.

As compared with ascorbate, dehydroascorbate supported near maximal lysyl hydroxylation but only minimal prolyl hydroxylation (10%) by enzymes from normal cells (Table V). However, with dehydroascorbate the rate of collagen lysyl hydroxylation by mutant enzyme was only 50–60% of the rate with ascorbate. Increasing the dehydroascorbate concentration from 0.5 mM to as high as 2.5 mM did not increase the rate of lysyl hydroxylation by either normal or mutant enzymes. When DTT (100 μM) was omitted from the assay mix-
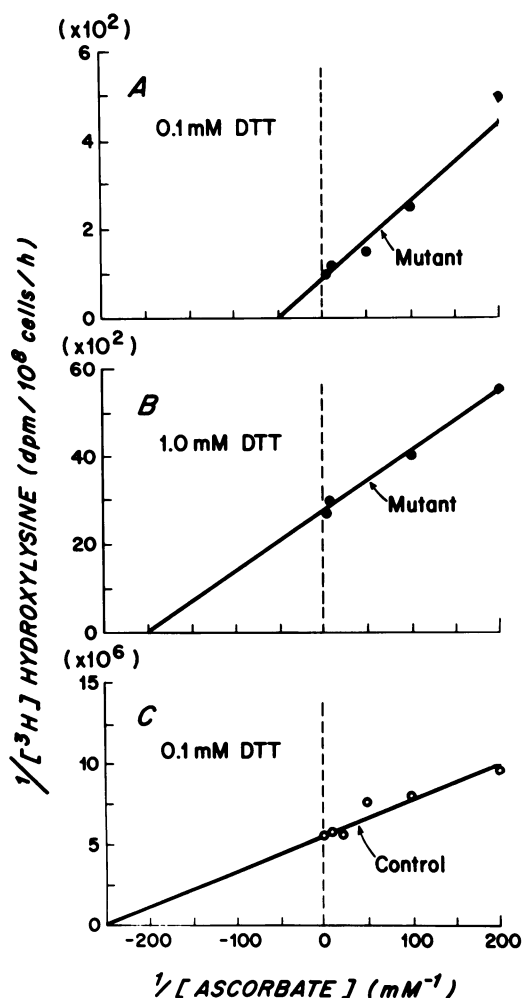

FIGURE 3 Double reciprocal plot of initial rate of collagen lysyl hydroxylation and ascorbate concentration. (A) Mutant enzyme assayed in 0.1 mM DTT. (B) Mutant enzyme assayed in 1.0 mM DTT. (C) Control enzyme assayed in 0.1 mM DTT.

TABLE V

Effect of Ascorbate, Dehydroascorbate, and Iron on Activities of Collagen Hydroxylases

Cells	Fe(II)	Fe(III)	(Ascorbate)	(Dehydro-ascorbate)	Hydroxylase activity	
					Lysyl	Prolyl
	mM	*mM*	*mM*	*mM*	*dpm* $\times$ $10^{-6}/10^8$ *cells/h*	
Mutant 2	0.1	0	0.5	0	0.127	0.926
	0	0	0.5	0	0.075	0.842
	0	0.1	0.5	0	0.104	1.15
	0.1	0	0	0.5	0.068	0.199
	0	0.1	0	0.5	0.077	0.107
Control	0.1	0	0.5	0	0.337	1.37
	0	0	0.5	0	0.277	0.968
	0	0.1	0.5	0	0.319	1.29
	0.1	0.1	0.5	0	0.331	1.29
	0.1	0	0	0.5	0.284	0.095
	0	0.1	0	0.5	0.332	0.081

ture, both wild-type and mutant enzymes exhibited little activity with dehydroascorbate. Colorimetric assay indicated no detectable ascorbate in dehydroascorbic acid (500 μM in 40 mM Tris-HCl, pH 7.2) but when DTT was added to the solution (to 100 μM), the ascorbate concentration rose to 10 μg/ml (approximately 60 μM). Thus it appeared that prolyl hydroxylase might have a considerably higher apparent K_m for ascorbate than either normal or mutant lysyl hydroxylase. Measurement of the apparent K_m of prolyl hydroxylase for ascorbate (100 μM) confirmed this prediction (Fig. 4).

α-Ketoglutarate. Lysyl and prolyl hydroxylases from normal and mutant cells required α-ketoglutarate in the assay solutions for the hydroxylation reactions to occur at significant rates. The lysyl hydroxylases in wild-type and mutant cells had similar affinities for α-ketoglutarate (apparent K_m 20 μM) (Fig. 5A and B). The apparent K_m of prolyl hydroxylases from wild-type and mutant cells was 4 μM, and the Lineweaver-Burk plot indicates substrate inhibition of prolyl hydroxylase at α-ketoglutarate concentrations above 100 μM (Fig. 6). Though the activities of prolyl and lysyl hydroxylases were coordinately inhibited (70–96%) by 5 mM α-ketoglutarate, with 1 mM α-ketoglutarate prolyl hydroxylase activity was selectively inhibited 80–90%, while lysyl hydroxylase activity was affected only slightly.

Iron. Although crude cell lysates of normal and mutant fibroblasts required ferrous ion in assay solutions for collagen hydroxylations to occur at maximal rates, in the absence of this cofactor considerable activity was still observed (Table V). Ferric chloride did not inhibit either lysyl or prolyl hydroxylase and enzyme activities were about the same in either ferric chloride (100 μM) or ferrous sulfate (100 μM). Reduction of Fe(III) to

Fe(II) by ascorbate or DTT could be responsible for the observed effects.

Other properties of normal and mutant enzymes

Aggregation in low ionic strength buffers. The ionic strength of the extracting buffer had little effect on the solubility of lysyl hydroxylases in the 50,000 *g* supernate of cell lysates (see above), but molecular sieve chromatography indicated that the lysyl hydroxylase from normal cells formed high molecular weight aggregates in low ionic strength buffers. Lysyl hydroxylase activity was readily separable from prolyl hydroxylase activity when normal cell lysates (prepared in 40 mM Tris-HCl, 250 mM sucrose, 50 mM 2-ME with or without 0.1% Triton X-100) were chromatographed on a
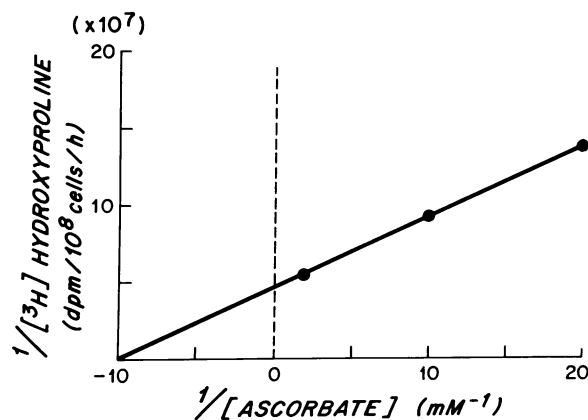

FIGURE 4 Double reciprocal plots of initial rate of collagen prolyl hydroxylation and ascorbate concentration. The data shown are those obtained with enzyme from wild-type cells, but an identical apparent K_m was measured for enzyme in mutant cells.
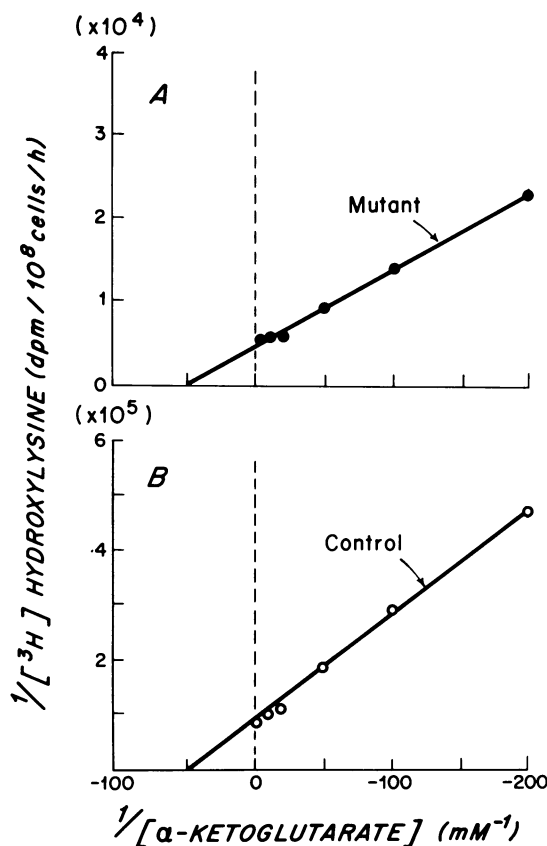
FIGURE 5 Double reciprocal plots of α-ketoglutarate concentration and initial rate of collagen lysyl hydroxylation by enzymes in mutant (A) and control cells (B).

Bio-Gel A-1.5 column equilibrated and eluted with 50 mM Tris-HCl, pH 7.4, 10% glycerol. In this low ionic strength buffer, lysyl hydroxylase activity in normal cells was eluted in the region of the excluded volume, but this activity in mutant cells was present almost exclusively
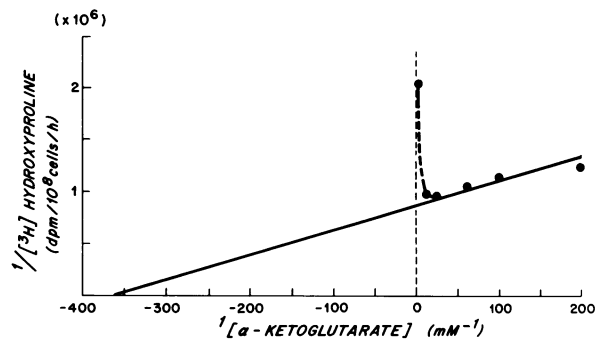

FIGURE 6 Double reciprocal plot of initial rate of collagen prolyl hydroxylation and α-ketoglutarate concentration. The data shown are obtained from studies of the enzyme in mutant cells, but the enzyme in control cells gave identical results.
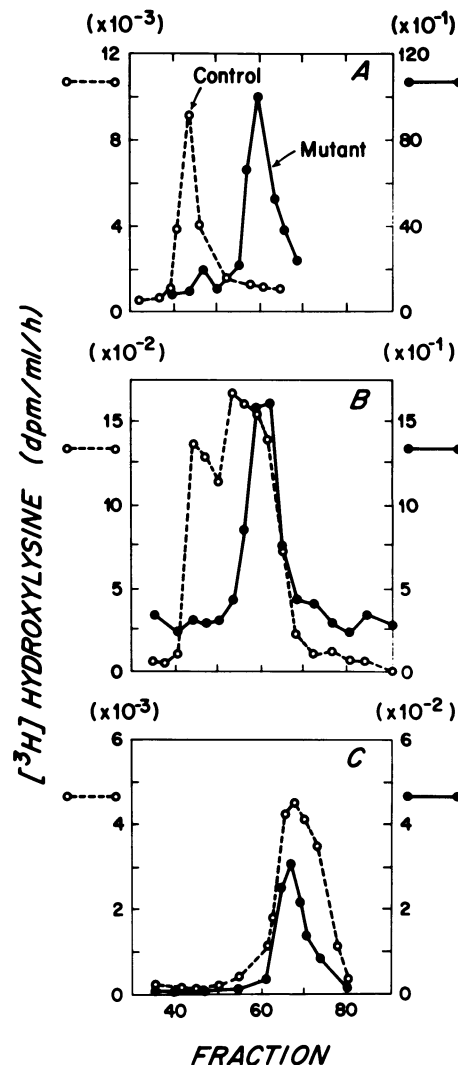

FRACTION

FIGURE 7 Gel chromatography of lysyl hydroxylases in control and mutant cells. Bio-Gel A—1.5 column, 1.5 × 81 cm, 1.2 ml fractions collected. (A) Buffer was 50 mM Tris-HCl, pH 7.4, 10% glycerol. (B) Buffer was 100 mM Tris-HCl, pH 7.2, 50 mM 2-ME. (C) Buffer was 50 mM Tris-HCl, pH 7.4, 10% glycerol, 150 mM NaCl.

in fractions corresponding to a lower molecular weight (Fig. 7A). Peak prolyl hydroxylase activity from both normal and mutant cells was eluted in fraction 60. Catalase (used to calibrate the column) was eluted in fractions 68-75. Lysyl hydroxylase activity in control cell lysates chromatographed in higher ionic strength buffer (100 μM Tris-HCl, pH 7.2, 50 mM 2-ME) was eluted as two distinct but overlapping peaks, one in the region of high molecular weight aggregates excluded from the gel and the other in fractions preceding those containing prolyl hydroxylase activity. In 50 mM Tris-HCl, 10% glycerol, 150 mM NaCl, lysyl hydroxylase activity in

TABLE VI

Effect of Incubation Temperature on Activity of Collagen Hydroxylases

| Cells | Temperature | Hydroxylase activity | | | |
		Lysyl	Maximal activity	Prolyl	Maximal activity
	°C	$dpm \times 10^{-5}/10^6$ cells/h	%	$dpm \times 10^{-5}/10^6$ cells/h	%
Mutant 1	20	0.189	69	0.699	28
	25	0.258	94	1.02	41
	30	0.273	100	1.87	76
	34	0.168	61	2.21	89
	37	0.087	32	2.48	100
	40	0.066	24	2.20	89
	45	0.010	4	1.51	61
Control	20	0.453	34	0.395	26
	25	0.950	71	0.592	39
	30	1.28	97	1.24	83
	34	1.30	100	1.41	95
	37	1.14	88	1.49	100
	40	0.980	74	1.37	91
	45	0.472	20	0.99	66

normal cells was eluted in fractions corresponding to a low molecular weight component. Aggregation of prolyl hydroxylase was not influenced by ionic strength since in every buffer prolyl hydroxylase activity was eluted in identical fractions.

Thermal stability. Lysyl hydroxylase activity in lysates of normal and deficient cells was diminished by approximately 95% after heating the enzyme (in solution with cofactors except for collagen substrate) 5 min at 60°C. After 15 min at 50°C, approximately 35% of the activity of the enzymes from both sources was retained. At lower temperatures the relative instability of the mutant enzyme was apparent. Though lysyl hydroxylase activity in lysates of normal cells was maximal when the assay mixtures were incubated at 34°C (for 60 min), approximately the same activity was measured after incubation at 30°, 34°, and 37° C (Table VI). Lysyl hydroxylase from mutant cells exhibited peak activity when incubation was at 30°C, and incubation at 37°C resulted in a 68% decrease in the rate of collagen lysyl hydroxylation. Prolyl hydroxylase from both normal and mutant cells exhibited maximal activity when incubation was at 37°C.

DISCUSSION

The finding that skin fibroblasts from siblings with hydroxylysine-deficient collagen (Ehlers-Danlos syndrome, type VI) contained much lower collagen lysyl hydroxylase activity than cells from normal subjects offered an experimental system amenable to further study of the nature of the defect. Diminished enzyme activity could conceivably be due to: (*a*) a normal enzyme in normal amount but in an environment where it is inactive (e.g. combined with another protein or attached to a membrane); (*b*) a normal or abnormal enzyme present in subnormal amount as a result of decreased synthesis, increased degradation, or defect in activation of a proenzyme; (*c*) a normal enzyme present in normal amount but less active due to the presence of inhibitors or absence of activators; (*d*) an abnormal enzyme with altered kinetic properties. We have been able to provide some data regarding these possibilities. Our studies indicate that normal amounts of enzyme activity cannot be extracted from mutant cells. Diminished lysyl hydroxylase activity is evident in whole cell sonicates and in the 50,000 *g* supernates of mutant cell lysates in low or high ionic strength buffers or in buffer containing detergent. A small amount of lysyl hydroxylase activity is found in sedimentable fractions of cell sonicates and can be solubilized by extraction of acetone powders of these fractions; however, the activity in the mutant cells is proportionally reduced in each of these fractions.

Studies of collagen prolyl hydroxylase in L-929 mouse fibroblasts indicated that during early log phase of culture growth, ascorbate may play a role in activation of prolyl hydroxylase (9). We found prolyl hydroxylase activity was decreased in ascorbate-supplemented wild-type or mutant human fibroblast cultures, but since lysyl hydroxylase activity was unaffected, or slightly elevated, the net result was an increase in the ratio of lysyl hydroxylase activity to prolyl hydroxylase activity. Our data concerning the effect of ascorbate on hydroxylase

activities in human skin fibroblasts cannot be directly compared with the studies of L-929 cells. We did not examine the effect on hydroxylase activities 1 h after administration of ascorbate to cultures in early log phase of growth; rather, we studied the effects of ascorbate supplementation throughout culture growth to confluence.

The results of studies of lysyl hydroxylase activity in mixtures of normal and mutant cell lysates indicate neither inhibition of enzyme in normal cells by material in mutant cells nor activation of enzyme in mutant cells by lysate of normal cells. A number of our observations suggest that the most probable reason for low lysyl hydroxylase activity in mutant cells is an alteration in the primary structure of the enzyme. Since all properties of lysyl hydroxylase from the two siblings were identical, we assume the same altered enzyme is present in skin fibroblasts from both individuals. The enzyme from deficient cells is less stable at 37°C than the normal enzyme. Mutant and wild-type enzymes show differences in aggregation properties: the wild type forms high molecular weight aggregates in low ionic strength buffers, but the mutant enzyme does not. In some experiments, after dialysis against buffer containing 10 mM DTT, the activity of the mutant enzyme was stimulated to a level comparable to that of the normal enzyme. These data suggest that the defective enzyme protein is present in deficient cells, and although the mutant enzyme is relatively inactive under conditions where the normal enzyme is fully active, under certain conditions it can be activated. Although mutant and wild type lysyl hydroxylases have identical affinities for α-ketoglutarate and exhibit a similar requirement for either Fe(II) or Fe(III), in the presence of 100 μM DTT the mutant enzyme has an apparent K_m for ascorbate approximately five times that of the normal enzyme. The concentration of DTT present in the assay solution (0.1 or 1.0 mM) had no effect on the apparent K_m of the normal enzyme for ascorbate, but in 1 mM DTT the apparent K_m of the mutant enzyme approached that of the normal. However, since assays were performed with an ascorbate concentration well in excess of the apparent K_m of the mutant enzyme, the stimulation of activity by DTT must have an additional explanation, which we cannot provide at this time. We also note that interpretation of all of our kinetic studies must take into account the use of underhydroxylated collagen substrate at less than saturating concentrations. However, since all of the apparent K_m's were determined from Lineweaver-Burk plots, which were linear, it is likely that the analyses are valid.

Hydroxylases in buffer without 2-ME are inactive unless DTT is present in the assay mixture. Furthermore, PCMB is inhibitory. It would thus appear, as in chick embryo enzymes (11, 18), that sulfhydryl groups are necessary for activity. However, lysyl and prolyl hydroxylases in crude lysates of wild-type or mutant human skin fibroblasts are relatively stable in high concentrations of sulfhydryl reagents (10 mM DTT or 50 mM 2-ME), although collagen hydroxylases from chick embryos are known to be inactivated by such concentrations of sulfhydryl compounds (11, 18). Although we found some loss of prolyl and lysyl hydroxylase activity in human cells incubated with 1 mM DTT in the culture medium, incubation of L-929 cells with this concentration of DTT caused loss of activity interpreted as due to disaggregation of prolyl hydroxylase into inactive subunits (9). It would appear that collagen hydroxylases from different sources may have different characteristics.

As yet we have little information that would help to explain the variation in hydroxylysine content of collagens from different tissues of the two subjects studied here. The existence of multiple forms of lysyl hydroxylase in various tissues remains a possibility, although other explanations are also likely. Lysyl and prolyl hydroxylases cannot act upon their specific residues if the component collagen polypeptides are in triple helical conformation (19–21). Therefore additional factors controlling lysyl hydroxylation could involve the relative rate of prolyl hydroxylation, the rate of chain synthesis, association, and helix formation, and the intracellular sites where these reactions take place. Recent evidence suggests that the time of chain association can be different in different tissues. Chain association and helix formation in chick embryo cartilage cells apparently occur at a later stage than in chick embryo tendon cells; the lateness of chain association in cartilage cells could be one of the factors influencing the high hydroxylysine content of cartilage collagen (22).

Cofactor concentrations may also regulate the activities of collagen lysyl and prolyl hydroxylases and could therefore play a role in determining the extent to which lysine residues are hydroxylated, particularly if in some tissues these hydroxylations occur in different cellular compartments. Even though lysyl and prolyl residues are simultaneously hydroxylated in the same cellular location, the relative activities of the two enzymes would not necessarily be coupled. We found that 1 mM α-ketoglutarate almost completely inhibited prolyl hydroxylase, while lysyl hydroxylase activity was diminished only slightly. At low α-ketoglutarate concentrations the opposite effect occurs, since prolyl hydroxylase has greater affinity for this cofactor. Ascorbate concentration could also regulate the relative activities of the two hydroxylases, since prolyl hydroxylase has a significantly lower affinity for ascorbate than either normal or mutant lysyl hydroxylase. The difference in apparent K_m for ascorbate of prolyl and lysyl hydroxylases may be characteristic of enzymes from other sources, since

it has been shown that ascorbate-deficient 3T6 mouse embryo cells synthesize collagen severely deficient in hydroxyproline but only moderately deficient in hydroxylysine (23).

The rate and sequence of events in collagen synthesis and cofactor concentrations and therefore the relative activities of prolyl and lysyl hydroxylases may vary from tissue to tissue. It is possible that all tissues of individuals with hydroxylysine-deficient collagen contain a mutant lysyl hydroxylase with altered kinetic properties, but the hydroxylysine content of the collagens synthesized by cells of different origin need not be reduced to the same extent. In this respect it would be important to determine if all subjects with the disorder show a similar reduction in the level of lysyl hydroxylation in collagens from different tissues. Though hydroxylysine-deficient skin collagen and low skin fibroblast lysyl hydroxylase activity has been established in another subject with Ehlers-Danlos syndrome type VI (24), information is not yet available concerning the hydroxylysine content of other tissues. Further studies of collagen hydroxylase activities may indicate the reasons for a variability in the hydroxylysine deficiency of these collagens. The decreased extent of hydroxylation must be critical in the alteration of mechanical properties of the collagen from different tissues, which must, in turn, be responsible for the abnormal phenotype.

ACKNOWLEDGMENTS

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