Inhibition of prolyl 4-hydroxylase by oxalyl amino acid derivatives *in vitro*, in isolated microsomes and in embryonic chicken tissues

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The potency of oxalyl amino acid derivatives as inhibitors of prolyl 4-hydroxylase was studied *in vitro*, in isolated microsomes and in chicken embryonic-tissue culture. These compounds represent structural analogues of 2-oxoglutarate in which the $-\text{CH}_2-$ moiety at C-3 is replaced by $-\text{NH}-$, with or without further structural modifications. The most efficient inhibitor of purified prolyl 4-hydroxylase was oxalylglycine. Its mode of inhibition was competitive with respect to 2-oxoglutarate. The K_i value varied between 1.9 and 7.8 μM , depending on the variable substrate used. Oxalylalanine inhibited purified enzyme with a K_i

of 40 μM . Other oxalyl amino acid derivatives showed little inhibitory activity. In microsomes isolated from embryonic chicken bone, oxalylglycine and oxalylalanine inhibited prolyl hydroxylation with IC_{50} values of 23 and 120 μM respectively. Dimethyloxalylglycine was not an inhibitor of purified prolyl 4-hydroxylase and only weakly active in the microsomal system, but efficiently suppressed hydroxyproline synthesis in embryonic chicken calvaria and lung. The data suggest that dimethyloxalyl amino acids are converted into active inhibitors in intact cells, most likely in the cytoplasmic compartment.

INTRODUCTION

Inhibitors of purified prolyl 4-hydroxylase (EC 1.14.11.2) have received much interest as potential fibrosuppressive agents, due to the key role of this enzyme in collagen biosynthesis [1–3]. Pyridine-2-carboxylates [4], 3,4-dihydroxybenzoic acid and related compounds [5], as well as oxaproline peptides [6], were shown to act as analogues of the enzyme's substrates 2-oxoglutarate, ascorbate and protocollagen *in vitro* respectively. However, their IC_{50} values as inhibitors of cellular hydroxyproline formation was several orders of magnitude higher compared with those obtained in studies of the purified enzyme. The difference in efficiency was attributed mainly to poor membrane permeation [7–9].

Conversion of the polar acidic inhibitors 3,4-dihydroxybenzoate and pyridine-2,4-dicarboxylate into the respective non-polar ethyl esters improves membrane permeation [8,10]. Ethyl 3,4-dihydroxybenzoate [8,11], but not diethyl pyridine-2,4-dicarboxylate [10,12], is an inhibitor of purified prolyl 4-hydroxylase. Specialized tissues like embryonic chicken calvaria are able to convert diethyl pyridine-2,4-dicarboxylate into an actively inhibitory substance [10]. This conversion is thought to take place in the cytosolic compartment [10], because the free acid, but not its diethyl derivative, is a potent inhibitor of prolyl hydroxylation in purified microsomes [12].

In the present study, we have investigated the potency of several oxalyl amino acids and their dimethyl derivatives as inhibitors of purified chicken prolyl 4-hydroxylase, as well as hydroxyproline formation in microsomes and in embryonic chicken tissues. *N*-Oxalylglycine has been described as inhibitor of the activity of purified, but not cellular, prolyl 4-hydroxylase [13,14]. By contrast, we have now found that dimethyloxalyl amino acids are inhibitors of cellular hydroxyproline formation in embryonic chicken tissues.

MATERIALS AND METHODS

Source of compounds

(Pro-Pro-Gly) $_{10}$ ·9H $_2$ O, the synthetic substrate for prolyl 4-hydroxylase, was obtained from the Protein Research Foundation, Minoh, Osaka, Japan. 2-Oxo[5- ^{14}C]glutarate was obtained from Amersham Buchler, Braunschweig, Germany. It was diluted to 2×10^7 d.p.m./ μmol by mixing with unlabelled compound purchased from Merck, Darmstadt, Germany. [3,4- ^3H]Proline and [U- ^{14}C]proline were purchased from New England Nuclear, Dreieich, Germany. 2,4-Dinitrophenylhydrazine (2,4-DNPH) and L-hydroxyproline were obtained from E. Merck, Darmstadt, Germany. Catalase was purchased from Sigma, St. Louis, MO, U.S.A. Dulbecco's medium (DMEM) and BSA were purchased from Serva, Heidelberg, Germany. All other substances were obtained from Sigma, Munich, Germany, or Merck.

Synthesis of oxalyl amino acid derivatives

The synthesis of dimethyl oxalyl amino acids and oxalyl amino acids was performed as shown in Scheme 1. Oxalic acid monomethyl ester chloride (1 equiv.) was added dropwise to a mixture of amino acid methyl ester hydrochloride (1 equiv.), triethylamine (2 equiv.), and 4-dimethylaminopyridine (2 equiv.) in dichloromethane at room temperature. The reaction mixture was stirred at the same temperature for 12 h and poured into a solution of aq. satd. NaHCO $_3$. The organic layer was separated and washed with aq. satd. NaCl, dried over anhydrous Na $_2$ SO $_4$, and evaporated. The oily products were purified by chromatography with ethyl acetate/methanol (5:1, v/v) over silica to give the oxalic amino acid methyl esters.

A 100 mmol portion of 1 M NaOH was added dropwise to a solution of 48 mmol of methyl ester in methanol (500 ml) at

Scheme 1 General structural formulae and synthesis of oxalyl amino acid derivatives

R represents -H or -CH₃, R' the respective amino acid side chain.

0–10 °C for 1 h. The mixture was stirred for 1 h at room temperature, evaporated in a vacuum and dried in a high vacuum. The residue was stirred with diethyl ether (300 ml). The solid was collected by suction filtration and dried for 4 h in vacuum. The structures of all compounds were confirmed by ¹H-n.m.r. and m.s.

Purification of enzyme and substrate

Prolyl 4-hydroxylase was purified from homogenized 14-day-old-chick embryos by (NH₄)₂SO₄ fractionation (0–70% satn.), affinity chromatography on poly-L-proline coupled to Sepharose 4B and DEAE-cellulose chromatography as previously reported [15,16]. [3,4-³H]Proline-labelled procollagen substrate was obtained by incubating calvaria from 14-day-old-chick embryos in the presence of [3,4-³H]proline and 2,2'-dipyridyl as previously described [17].

Enzyme assays

Determination of enzyme activity was carried out as described previously [18]. In short, 0.02–0.03 µg of prolyl 4-hydroxylase was incubated in 0.05 ml of 0.04 M Tris/HCl, pH 7.5, containing 0.05 mM FeSO₄, 0.1 mM 2-oxo[5-¹⁴C]glutarate (100000 d.p.m.), 20 µg (0.4 mg/ml) of (Pro-Pro-Gly)₁₀, 1 mM ascorbate, 0.4 mg of catalase/ml, 0.5 mM dithiothreitol and 2 mg of BSA/ml. The peptide substrate was denatured at 100 °C immediately before addition to the incubation mixture. The samples were then incubated at 37 °C for 40 min. A 25 µl portion of a solution containing 20 mM non-labelled succinate and 2-oxoglutarate, serving as carrier for the radioactivity, and 25 µl of 0.16 M 2,4-DNPH in 30% (w/v) HClO₄ were added. The precipitation reaction was allowed to proceed for 30 min at room temperature, followed by addition of 50 µl of 1 M 2-oxoglutarate and further incubation for 30 min. The samples were then centrifuged at 3000 g for 5 min. A 100 µl portion of the supernatant was transferred into liquid-scintillation vials and the radioactivity was determined.

Inhibitors to be tested were added at between two and four fixed concentrations, and the concentration of 2-oxoglutarate was varied from 0.02 to 0.08 mM. All inhibitor stock solutions were neutralized with NaHCO₃ before addition to the incubation mixture. The K_i values were calculated from the secondary transforms of the primary plots.

The inactivation experiment was carried out according to an established method [19]. The formation of hydroxyproline in the presence of oxalylglycine instead of 2-oxoglutarate was studied in a reaction using 200000–300000 d.p.m. of [3,4-³H]proline-

labelled procollagen in the presence of either 0.02–0.08 mM unlabelled 2-oxoglutarate or 0.01–0.5 mM oxalylglycine. The concentrations of the other components were identical with those outlined above. Hydroxyproline formation was assayed as described previously [17].

Microsomal proline hydroxylation assay

The purification of microsomes from embryonic chicken bone after metabolic labelling with [4-³H]proline in the presence of the prolyl hydroxylase inhibitor 2,2'-dipyridyl, and the experiments controlling the intactness of the prepared microsomes, were carried out as described previously [12,20]. The microsomes were stored as frozen pellets in liquid N₂ and were thawed and suspended in 0.25 M sucrose/0.05 M Tris/HCl/1 mM MgCl₂/5 mM KCl, pH 7.6, immediately before the experiments were conducted. The activity of prolyl 4-hydroxylase in intact microsomes was measured as release of ³H₂O from the intracisternal underhydroxylated procollagen. In the standard assay, microsomes were incubated for 20 min at 37 °C in a reaction mixture containing 0.1 mM 2-oxoglutarate, 0.05 mM Fe₂SO₄, 0.5 mM L-ascorbate, 0.1 mM thymol and 0.1 mM dithiothreitol, as well as various concentrations of oxalylglycine, oxalylalanine or their dimethyl derivatives. The total reaction volume was 0.1 ml. The amount of ³H₂O released was measured as described previously [17].

In a second set of experiments, prolyl 4-hydroxylase was released from the isolated microsomes prior to the experiments by treatment with 0.1% Brij-35 and 0.6 M NaCl as described previously [12]. The further assay was identical with the procedure described above.

Labelling of the chick embryo calvaria

Calvaria were removed from chicken embryos, aged 15 days, and washed for 3 min in DMEM at 37 °C. They were then transferred into Pyrex tubes (eight calvaria/tube) containing 3 ml of medium, 6 µCi of [U-¹⁴C]proline, and various concentrations of oxalyl amino acid derivatives. The samples were incubated for 1–4 h at 37 °C. The experiment was terminated by placing the tubes in ice and separating the calvaria from the culture medium. The calvaria were washed once with 3 ml of fresh medium, which was then pooled with the incubation medium. BSA and phenylmethanesulphonyl fluoride (PMSF) were added (final concns. 1 mg and 6 µg/ml respectively). The calvaria were extracted for 16 h with 25 ml of 0.5 M acetic acid.

The following procedure was identical for the medium and the calvaria extracts. The samples were extensively dialysed against 0.5 M acetic acid at 4 °C. Aliquots were withdrawn for SDS/PAGE, and the remaining material was freeze-dried, resuspended in 2 ml 6 M HCl and hydrolysed at 105 °C for 25 h. After evaporation of the acid the samples were dissolved in 2 ml of water. Underivatized [¹⁴C]proline and [¹⁴C]hydroxyproline were separated by h.p.l.c. using a Bio-Rad HPX-87C column (300 mm × 7.8 mm) operated at 65 °C and a flow rate of 0.8 ml/min. The mobile phase consisted of 0.01 M calcium acetate, pH 5.5. Radioactivity was detected using a flow-through radioactivity monitor (LB 505; Berthold, Wildbad, Germany) equipped with a GT 400 U4 cell. Under these conditions, hydroxyproline was eluted from 18.1 to 24.7 min (maximum at 19.8 min) and proline from 28.7 to 36.9 min (maximum at 30.6 min). The position of the proline peak was verified by application of commercially available [U-¹⁴C]proline, and the position of the hydroxyproline peak by chromatography of the non-labelled amino acid in combination with u.v. detection

(results not shown). Inhibition of hydroxyproline synthesis is calculated according to the formula:

$$\text{Inhibition (\%)} = 100 - \left[\frac{100 \times \text{peak area (Hyp)}}{\text{peak area (Hyp + Pro)}} \right]$$

Chicken embryonic lung was obtained by dissecting 19-day-old chicken embryos at the level of the diaphragm. Macroscopically visible parts of the tracheal and bronchial system were removed. Incubation with [^{14}C]proline was carried out as described for the calvaria. The synthesis of hydroxyproline was assayed according to the method described by Juva and Prockop [21].

SDS/PAGE

Freeze-dried samples were reconstituted with buffer containing 0.01 M Tris/HCl, pH 8, 1 mM EDTA, 1% SDS and 5% 2-mercaptoethanol. The material was denatured at 95 °C for 10 min; 20000–50000 d.p.m. of [^{14}C]-labelled protein/sample was run on a 1.5 mm $\times$ 150 mm 5–10% gradient PAGE/SDS gel [22]. After fixation in methanol/acetic acid/water (3:1:7, by vol.), the gels were processed for fluorography by the Bonner–Laskey method [23]. The dried films were exposed to Kodak X-Omat films at –70 °C. The individual species were identified according to previously published data [10,24–26].

RESULTS

Enzyme assays

Underivatized oxalyl amino acids proved to be inhibitors of purified prolyl 4-hydroxylase. Oxalylglycine, the most potent compound in the series, showed a competitive type of inhibition when 2-oxoglutarate was used as variable substrate, with a K_i value of $1.9 \pm 0.2 \mu\text{M}$ ($n = 3$). However, higher K_i values were observed when Fe^{2+} , ascorbate or the peptide substrate were used as variable substrates. The K_i value determined under these conditions was $7.0 \pm 3.1 \mu\text{M}$ ($n = 7$). The possible reasons for this discrepancy are discussed below. Oxalylglycine did not cause irreversible inhibition of the purified enzyme when tested in concentrations up to 0.5 mM. It was unable to support the hydroxylation of ^3H -labelled procollagen substrate over the same dose range (result not shown).

Oxalylalanine, carrying a methyl group at the carbon atom corresponding to C-4 of the 2-oxoglutarate backbone, was only slightly less effective, its K_i being $40 \mu\text{M}$. The other oxalyl amino acids used in this study, containing large side chains at C-4, substituents at the $-\text{NH}-$ group (corresponding to C-3 of 2-oxoglutarate) or a backbone of six atoms, were much less effective as inhibitors of purified prolyl 4-hydroxylase. Not all of them showed competitive inhibition with respect to 2-oxoglutarate. The data are summarized in Table 1. None of the studied dimethyl derivatives was an inhibitor of the purified enzyme at a concentration of 10 mM or below.

Studies in isolated chicken embryo microsomes

Oxalylglycine and oxalylalanine caused 50% inhibition of microsomal prolyl 4-hydroxylase activity at concentrations of 23 and 123 μM respectively. Very similar values were obtained when the enzyme was solubilized from microsomes by treatment with 0.1% Brij-35 and 0.6 M NaCl (Table 2), indicating that the microsomal membrane does not restrict the access of either compound to the enzyme. The relative efficacy of these compounds compares well with the K_i values obtained in the purified-enzyme studies (7 and 40 μM respectively). Both

dimethyl derivatives inhibited microsomal prolyl hydroxylation only at much higher concentrations (Table 2).

Chicken embryo calvaria studies

Dimethyloxalylglycine caused 50% inhibition of hydroxyproline formation in chicken embryo calvaria at a concentration of 2 μM

Table 1 Inhibition of purified prolyl 4-hydroxylase by oxalyl amino acids

Determination of K_i values was carried out as described in the Materials and methods section. Abbreviations: C, competitive mode of inhibition; NC, non-competitive mode of inhibition; N.D., not determined.

Oxalyl amino acid	K_i (mM)	Mode of inhibition versus 2-oxoglutarate
Oxalylglycine	0.0019–0.0070	C
Oxalylalanine	0.04	C
Oxalylsarcosine	1.0	NC
Oxalylcystine	1.25	C
Oxalyl- β -alanine	1.4	C
Oxalylvaline	> 10	N.D.
Oxalylproline	> 10	N.D.

Table 2 Effect of oxalyl amino acids and their dimethyl derivatives on prolyl hydroxylation by intact microsomes and solubilized enzyme

The experiments were carried out as described in the Materials and methods section. In the control samples used to demonstrate intactness of microsomes, 4.9% of the total radioactivity were found in the supernatant and 95.1% in the pelleted microsomes. After treatment with 0.1% Brij-35 and 0.6 M NaCl, 94.7% of the activity was found in the supernatant and 5.3% in the pellet. n is the number of experiments; each experiment involved duplicate samples. N.D., not determined.

Compound	IC_{50} value (μM)	
	Intact microsomes	Brij-35-treated microsomes
Oxalylglycine	23.3 ± 7.6 ($n = 3$)	18.7
Oxalylalanine	123 ± 34 ($n = 3$)	268
Dimethyloxalylglycine	540 ($n = 2$)	N.D.
Dimethyloxalylalanine	2600 ($n = 2$)	N.D.

Figure 1 Inhibition of hydroxyproline synthesis in chicken calvaria treated with dimethyloxalyl amino acids

Collagenous material was extracted from calvaria treated with dimethyloxalyl amino acids for 2.5 h in the presence of [^{14}C]proline, and the hydroxyproline content was analysed as described in the Materials and methods section. The inhibitors used were dimethyloxalylalanine ($\blacklozenge$) or dimethyloxalylglycine ($\diamond$). Error bars represent $\pm$ S.D. for four individual experiments.

Figure 2 Inhibition of hydroxyproline synthesis in chicken calvaria by incubation with dimethyloxallylglycine from 1 to 4 h

Collagenous material was extracted from calvaria treated with dimethyloxalyl amino acids in the presence of [¹⁴C]proline, and the hydroxyproline content was analysed as described in the Materials and methods section. (a) Total incorporation of radioactivity (○) and hydroxyproline synthesis (●) in uninhibited samples. (b) Effect of incubation with dimethyloxallylglycine. The concentration of inhibitor was 0 (○), 0.8 (●), 4 (▲), 20 (□) and 100 μM (■).

Table 3 Relative quantity of collagenous material produced by chicken calvaria in the presence of dimethyloxallylglycine

Chicken calvaria were incubated in the presence of [U-¹⁴C]proline and various concentrations of dimethyloxallylglycine as described in the Materials and methods section. An aliquot of the acid-extractable material was analysed by SDS/PAGE and densitometric evaluation of the autoradiographs. Values refer to the percentage of an individual peak of the total integrated area. Abbreviations: pro α1, pro α2, procollagen α1 and α2 chains; pC α1, pC α2, molecules of the α1 and α2 chains of procollagen type I lacking the C-terminal extension peptide; α1 and α2, collagen α1 and α2 chains. The amount of pro α2 and pC α2 species is summarized because they are not separated by electrophoresis if material treated with dimethyloxallylglycine is used.

Collagen species	[Inhibitor] (mM) ...	Relative quantity (%)				
		0	0.0008	0.004	0.02	0.1
pro α1		16.6	20.3	43.2	55.3	51.9
pC α1		19.0	14.2	5.4	2.3	2.4
pro α2		19.9	30.1	42.1	39.8	43.3
+ pC α2						
α1		31.2	24.4	6.4	—	—
α2		13.3	11.0	2.9	2.6	2.4

(Figure 1). About 20% inhibition of hydroxyproline synthesis was found at 0.8 μM, and 10 μM caused more than 90% inhibition. Very little variation was observed between experiments (Figure 1) or when the duration of the incubation was varied between 1 and 4 h (Figure 2). The total incorporation of proline-derived radioactivity was not reduced by dimethyloxallylglycine at 0.2 mM and was reduced by 38% at a

Figure 3 Fluorography of [¹⁴C]proline-labelled collagenous material from chicken calvaria

Collagenous material was extracted from calvaria treated with dimethyloxalyl amino acids in the presence of [¹⁴C]proline for 2.5 h as described in the Materials and methods section. The collagen species were identified in agreement with previously published studies [18–20]. (a) Cultures treated with 0.0008 (lane 2), 0.004 (lane 3), 0.02 (lane 4) and 0.1 mM dimethyloxallylglycine (lane 5) in comparison with controls (lanes 1 and 6). (b) Cultures treated with 0.008 (lane 2), 0.04 (lane 3), 0.2 (lane 4) and 1 mM dimethyloxallylglycine (lane 5) in comparison with controls (lanes 1 and 6). For abbreviations, see Table 3.

concentration of 1 mM. The total incorporation of leucine was reduced by 15% at 1 mM. These data suggest that dimethyloxallylglycine does not interfere with the total biosynthesis of proteins, and thus cell viability, in chicken calvaria at concentrations that completely suppress hydroxyproline formation.

Besides dimethyloxallylglycine, only the alanine derivative showed any inhibition of hydroxyproline generation at concentrations up to 1 mM. Its IC₅₀ was 0.35 mM (Figure 1). No reduction of the incorporation of proline-derived radioactivity was caused by dimethyloxallylglycine and the other oxalyl amino acid derivatives under study.

The acid-extractable material from calvaria, consisting of processed and non-processed procollagen type I chains, was studied by SDS/PAGE, fluorography, and densitometry. When dimethyloxallylglycine was used as inhibitor, characteristic effects were observed (Figure 3a; Table 3): The amount of completely processed α-chains and partially processed procollagen species, accounting for over 75% of the total collagenous material in the controls, was decreased in a dose-dependent manner. The electrophoretic mobility of these species was unchanged. By contrast, an absolute and relative increase of the amount of unprocessed procollagen α1(I) and α2(I) chains was observed.

At a dimethyloxallylglycine concentration as low as 4 μM, more than 85% of the extracted collagenous material consisted of unprocessed procollagen chains. The electrophoretic mobility of both procollagen chains was increased in a dose-dependent way (Figure 3). The observed migration difference of the procollagen α1-chain was greater than that of the α2-chain

Table 4 Relative migration of procollagen chains produced by chicken calvaria in the presence of dimethyloxalylglycine

Chicken calvaria were incubated in the presence of [^{14}C]proline and various concentrations of dimethyloxalylglycine as described in the Materials and methods section. An aliquot of the acid-extractable material was analysed by SDS/PAGE and densitometric evaluation of the fluorographs. 'Migration difference' refers to the position of the procollagen species compared with the position of the same species in the control material.

[Inhibitor] (mM)	Migration difference (mm)	
	Procollagen $\alpha 1$ chain	Procollagen $\alpha 2$ chain
0	0	0
0.0008	0.7	0.5
0.004	1.7	1.0
0.02	2.2	1.6
0.1	2.4	1.8

Table 5 Effects of dimethyloxalylglycine treatment on the hydroxyproline synthesis in chicken embryonic lung tissue cultures

IC_{50} values refer to the concentration of inhibitor producing 50% inhibition of either hydroxyproline synthesis or total incorporation of proline. The results are expressed as d.p.m. of proline or hydroxyproline/g of lung tissue. The amount of tissue used per sample varied between 0.5 and 0.7 g. $\text{Hyp}_{\text{ex/in}}$ is the ratio of hydroxyproline detected in medium and tissue.

(a) Culture medium

[Dimethyloxalylglycine] (μM)	Radioactivity (d.p.m.)		Hyp/Pro ratio
	Hyp	Pro	
0	7554	48 623	0.183
2	4295	51 837	0.091
10	1840	42 205	0.047
50	1445	23 839	0.065
250	1337	13 932	0.105
IC_{50} (μM) ...	3.7	50	2.0

(b) Lung tissue

[Dimethyloxalylglycine] (μM)	Radioactivity (d.p.m.)		Hyp/Pro ratio	$\text{Hyp}_{\text{ex/in}}$
	Hyp	Pro		
0	20 309	830 332	0.025	0.37
2	22 358	935 879	0.024	0.19
10	8 924	904 093	0.010	0.21
50	3 447	589 294	0.006	0.42
250	3 071	350 907	0.009	0.44
IC_{50} (μM) ...	9.3	200	8.5	

(Table 4). A linear correlation between the migration difference and the inhibition of hydroxyproline synthesis was found. The correlation coefficients (r^2) were 0.993 and 0.975 for the procollagen $\alpha 1$ and $\alpha 2$ chains respectively.

The species of procollagen type I lacking the C-terminal extension peptide were not observed in controls or treated cultures, in agreement with a previous study [10].

Using dimethyloxalylalanine, similar effects were found, but only at much higher concentrations. The effects caused by 1 mM

Figure 4 SDS/PAGE of acid-extractable material from the culture medium of embryonic chicken lung cultures

The lanes represent chicken calvaria type I procollagen species (lane 1), material extracted from control cultures (lane 2), and material extracted from cultures treated with 2, 10, 50, and 250 μM dimethyloxalylglycine (lanes 3–6 respectively). The amount of material co-migrating with chicken calvaria procollagen type I is reduced in a dose-dependent manner.

of this compound were comparable with those caused by 2 μM dimethyloxalylglycine (Figure 3).

Dimethyloxalylglycine was also used as an inhibitor of hydroxyproline synthesis in embryonic chicken lung (Table 5). The absolute amounts of hydroxyproline extracted from tissue and culture medium were inhibited by 50% at a concentration of 9.3 and 3.7 μM respectively. When hydroxyproline synthesis is expressed as the Hyp/Pro ratio, the respective IC_{50} values were 8.5 and 2.0 μM . These values reflect a predominant reduction of extracellular hydroxyproline levels, as expressed by the $\text{Hyp}_{\text{ex/in}}$ ratio in Table 5. Dimethyloxalylglycine causes a dose-dependent decrease of material co-migrating with chicken calvaria type I procollagen in SDS/PAGE (Figure 4).

DISCUSSION

The present study characterizes oxalyl amino acids as inhibitors of purified and microsomal prolyl 4-hydroxylase, and dimethyloxalyl amino acids as inhibitors of hydroxyproline synthesis, in embryonic chicken tissue cultures.

The most potent inhibitor of purified prolyl 4-hydroxylase was oxalylglycine, a compound which differs from the enzyme's substrate, 2-oxoglutarate, only by replacement of the methylene group at C-3 by $-\text{NH}-$. Oxalylglycine inhibits purified prolyl 4-hydroxylase competitively with respect to the enzyme's co-substrate, 2-oxoglutarate, but cannot substitute for 2-oxoglutarate in the hydroxylation reaction, in spite of the presence of a 2-oxocarboxylic acid moiety, the prerequisite of decarboxylation, in the molecule. This finding strongly supports the interpretation of the decarboxylation reaction as a nucleophilic attack of an electron pair of the activated dioxygen molecule on the carbonyl carbon atom, C-2, of 2-oxoglutarate [1]. The p_z orbital of this atom is thought to contain little electron density, owing to the presence of the strongly electronegative environment. The neighbouring C-3 carbon atom is sp^3 -hybridized and cannot contribute electron density. In oxalylglycine, C-3 is replaced by the sp^2 -hybridized nitrogen atom. Its p_z orbital contains an electron pair that can contribute to the electron density of the C-2 p_z orbital, so that a nucleophilic attack may no longer be possible.

The compound's reported K_i was 0.54 μM in experiments with 2-oxoglutarate as a variable substrate [14]. The K_i value determined in corresponding experiments in the present study (1.9 μM) is only slightly higher and may reflect the limits of

experimental error. However, we have found consistently higher K_i values ($7.0 \pm 3.1 \mu\text{M}$) when Fe^{2+} , ascorbate or the peptide substrate were used as variable substrates.

In the 2-oxoglutarate decarboxylation assay of prolyl 4-hydroxylase, enzyme activity is measured by determining the rate of decarboxylation of ^{14}C -labelled 2-oxoglutarate. In Lineweaver-Burk-type enzyme kinetics experiments, the variable substrate is used under non-saturating conditions. In the experiments when 2-oxoglutarate is used as variable substrate, the concentration of radiolabelled substrate used for the determination of reaction velocity cannot be considered constant during the entire incubation period. By contrast, 2-oxoglutarate is used at saturating concentrations in all experiments with Fe^{2+} , ascorbate and peptide substrate as variable substrates. The observation of consistently higher K_i values under these latter conditions suggests that the potency of an inhibitor is overestimated in experiments where the variable substrate is identical with the substrate used for determination of enzyme activity.

The effects of dimethyloxalylglycine in the calvaria cultures suggests interference with the post-translational processing of procollagen type I. Suppressed cellular synthesis of hydroxyproline, inhibition of procollagen processing, the accumulation of procollagen chains and their atypical electrophoretic migration are typical consequences of inhibition of intracellular prolyl 4-hydroxylase [10].

Another consequence of inhibition of intracellular prolyl 4-hydroxylase is the retention of underhydroxylated collagen in the endoplasmic reticulum [7,10]. Collagen extracted from calvaria does not exclusively represent intracellular collagen, owing to the large amounts of cell-free matrix in this tissue. Intracellular retention of underhydroxylated procollagen therefore cannot be demonstrated by comparison of the amounts of hydroxyproline extracted from tissue and culture medium. By contrast, cell-free matrix is scarce in lung tissue. Accordingly, the ratio of extracellular versus intracellular hydroxyproline is decreased by dimethyloxalylglycine administration.

The behaviour of the aliphatic compound oxalylglycine and its dimethyl derivative parallels the behaviour of the known aromatic prolyl 4-hydroxylase proinhibitor/inhibitor pair diethyl pyridine-2,4-dicarboxylate/pyridine-2,4-dicarboxylate. Oxalylglycine potently inhibits purified and microsomal prolyl 4-hydroxylase. Its dimethyl derivative is not an inhibitor of the purified and microsomal enzyme, but is able to suppress prolyl hydroxylation in embryonic chicken calvaria and lung. It is therefore plausible

to assume that dimethyloxalylglycine is activated intracellularly in the same way that has been suggested for diethyl pyridine-2,4-dicarboxylate [10].

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