INHIBITION OF PROLYL HYDROXYLASE BY COLLAGEN

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SUMMARY

Heat-denatured collagens obtained from tissues of several vertebrate species strongly inhibited prolyl hydroxylase activity of both chick embryos and WI-38 fetal human lung fibroblasts. With 3,4-^{3}H-L-proline-labeled "protocollagen" ("deoxycollagen" chains) as substrate and one of several kinds of unlabeled or tritium-labeled collagens as inhibitor, the inhibition of hydroxylase activity was shown to be non-competitive. If such inhibition were to occur in vivo, it could represent a significant mode of regulation of prolyl hydroxylase activity or a means by which the degree of hydroxylation of nascent collagen is controlled.

INTRODUCTION

In the course of studying the activity of prolyl hydroxylase in WI-38 fibroblasts, particularly as the cells undergo successive doublings of population in culture, we observed that addition of heat-denatured crude preparations of fibroblast hydroxylase to active (i.e., unheated) preparations reduced hydroxylase activity. This inhibition could be largely prevented by pre-treatment of the heat-inactivated enzyme preparation with clostridial collagenase, inplicating accompanying collagen as the inhibitor. We then found that a variety of heat-denatured exogenous collagens could inhibit prolyl hydroxylase activity of chick embryos, WI-38 fibroblasts of several passage numbers, or of WI-38 VA132RA cells. The results of some of these studies are reported here together with a discussion of the possible function of collagen in regulation of hydroxylation of nascent collagen.

MATERIALS AND METHODS

3,4-^{3}H-L-Proline was obtained from New England Nuclear Corp.; a,a'-dipyridyl HCl from G.F. Smith Chemical Co.; gly-pro-ala, gly-pro-pro from Cyclo Chemicals; and gly-pro-hypro from Sigma Chemical Co. Highly purified clostridial collagenase (1) and collagens from carp swim bladder (ichthyocol), calf skin, rabbit skin and guinea pig skin were generous gifts from Drs. S. Takahashi and S. Seifter. The "core" of guinea pig

collagen, i.e. the portion of collagen that is retained by dialysis of collagen digested with collagenase, was prepared by a procedure similar to that described for the "core" of ichthyocol (2). Pure α_1 and α_2 chains of rat tendon tail were kindly supplied by Dr. O. Blumenfeld. Partially purified chick embryo prolyl hydroxylase was prepared according to Berg and Prockop (3). The pellet obtained between 30-65% saturated $(NH_4)_2SO_4$ was resuspended to a concentration of 15 mg protein per ml in the buffer suggested by those authors and dialyzed against the same buffer; this was the preparation used in these studies.

Preparation of 3,4-^{3}H-L-proline labeled protocollagen and collagen: Labeled protocollagen was prepared by incubating tibias from 11-day old chick embryos with ^{3}H-L-proline (35 mCi/μmole) in the presence of α,α'-dipyridyl and isolated using a published procedure (4) slightly modified. After incubation, the homogenized tibia suspension was centrifuged at 100,000 xg for 45 min, the supernate dialyzed against 20 mM tris-HCl at pH 7.6 and autoclaved for 20 min at 120° C. Insoluble material was removed by centrifugation and the supernate redialyzed against the same buffer. Using the same procedure, but omitting α,α'-dipyridyl and adding 0.5 mM L-ascorbate, 0.1 mM α-ketoglutarate and 0.1 mM Fe $(NH_4)_2(SO_4)_2$, 3,4-^{3}H-L-proline-labeled collagen was prepared. The ^{3}H-labeled protocollagen and collagen preparations respectively were digested with collagenase and fractionated on a Dowex 50 WX2 or Type P chromobeads ion exchange resin, Technicon Chemicals, S.A. column; in each case characteristic tripeptides were obtained.

Preparation of cell-free extract from WI-38 fibroblasts: WI-38 fibroblasts were grown in BME (Diploid) with Earle's salts (Gibco) containing 25 mM HEPES buffer, 50 μg/ml gentamycin (Schering), 2.5 μg/ml fungizone (Squibb) and 10% fetal calf serum (Flow Labs.). The final pH was 7.2. Cells were seeded in Blake bottles and refed every second day. After reaching confluency, the cells were detached by treatment with a solution containing 0.05% trypsin and 0.02% EDTA, harvested by centrifugation at 900 xg and washed 3 times with 100 volumes of a phosphate buffer-saline solution. Before and after centrifugation, counts were made of total and viable cells; cell viability was generally over 90%. Using a small volume of a buffer solution containing 50 mM HEPES at pH 7.0, 1 mM dithiothreitol and 10 μM EDTA, the final washed cell pellet was suspended to a protein concentration of 10 mg/ml (about 30 x 10^6 cells/ml). The cells were then disrupted by ultrasonication at 4° C for 15 sec at 40 W. Thus, crude extracts with relatively high specific activities of prolyl hydroxylase were obtained.

Assay of prolyl hydroxylase activity: Hydroxylation of the labeled proline residues in protocollagen was measured by detritiation of proline and release of the tritium into the aqueous medium (5). The reaction mixture contained in a final volume of 0.50 ml: Tris-HCl at pH 7.2, 25 μmoles; Fe $(NH_4)_2(SO_4)_2$, 0.05 μmole; L-ascorbic acid, 0.5 μmole; α-ketoglutaric acid, 0.05 μmole; bovine serum albumin, 1 mg; crystalline catalase (20 mg/ml), 10 μl; and 3,4-^{3}H-L-proline protocollagen (2.2 x 10^6 cpm/mg), 100 μl (350,000 cpm). Reactions were initiated by addition of 10-20 μl of chick prolyl hydroxylase preparation (15 mg/ml) or 10-40 μl of WI-38 fibroblast cell-free extract (10 mg/ml). Incubations were carried out at 30° C for 20 min and reactions were terminated by addition of 2.5 μmoles of α,α'-dipyridyl HCl. The assay mixture was then transferred to the side-arm of a Thunberg tube and the water collected by sublimation under high vacuum. Radioactivity was measured at 25-30% efficiency in a scintillation counting spectrometer. Activity is expressed as cpm of ^3H_2O released per 20 min per 0.5 ml of reaction mixture.

RESULTS AND DISCUSSION

Results summarized in Table 1 show that several heat-denatured collagens as well as separated α-chains strongly inhibited prolyl hydroxylase activity of chick embryos. Pretreatment of the exogenous collagen with collagenase significantly but not completely

TABLE I

The effect of collagens, collagen "core" and certain collagenolytic tripeptides on prolyl hydroxylase activity from chick embryos.

	Additions *	cpm of ^3H_2O released	% inhibition
Exp. I	None	2,445	--
	Ichthyocol, 128 µg	408	83
	Calf skin collagen, 83 µg	88	96
	Rabbit skin collagen, 93 µg	485	80
Exp. II	None	3,592	--
	Ichthyocol, 13 µg	2,362	35
	128 µg	702	81
	Guinea pig collagen, 10 µg	2,081	43
	100 µg	283	93
	Guinea pig collagen "core," 50 µg	3,176	12
Exp. III	None	3,370	--
	Ichthyocol, 128 µg	679	80
	Collagenase treated ichthyocol, ** 128 µg	2,549	25
Exp. IV	None	5,257	--
	Gly-pro-pro 0.4 µmole	4,924	6
	Gly-pro-ala 0.4 µmole	4,987	5
	Gly-pro-hypro 0.4 µmole	4,893	7
Exp. V	None	3,095	--
	Rat tendon tail α_1-chain 17 µg	1,341	57
	34 µg	825	73
	Rat tendon Tail α_2-chain 20 µg	1,965	37
	40 µg	1,398	55

* Aqueous solutions of the collagens (1 mg/ml) were prepared. Except for ichthyocol that was gelatinized at 50° C, all other collagens were autoclaved at 120° C for 15 min. Insoluble material was removed by centrifugation, and the protein concentration in the supernate was determined according to Lowry et al (6). The amounts of collagens are expressed as equivalents of serum albumin although, on a weight basis, collagens yield only about 50% of color given by albumin.

** 20 viscometric units (1) of purified clostridial collagenase were added to the ichthyocol solution in the presence of 50 mM Tris-HCl pH 7.6 containing 5 mM $CaCl_2$. After 24 hrs of incubation at 37° C, the enzyme was inactivated by boiling. It was then sonicated for 30 sec to obtain a homogeneous suspension, this was then added to the assay mixture for prolyl hydroxylase activity.

obviated this inhibition (Experiment III, Table 1). The residual inhibition was not due to the dominant tripeptides resulting from collagenolytic digestion as seen in Experiment IV of the table; nor was it due likely to the aggregate group of larger, more polar and per-

TABLE II

Inhibition of prolyl hydroxylase from chick embryos and from WI-38 fibroblasts by ^{3}H-labeled collagen

Substrate	cpm of $^{3}H_2O$ released by prolyl hydroxylase preparations from*		
	chick embryos	WI-38 fibroblasts**	
		control	ascorbic acid treated
1) tritiated protocollagen (2.2×10^6 cpm/mg, 0.15 mg)	5,765	1,754	3,371
2) tritiated collagen (2.0×10^6 cpm/mg, 0.15 mg)	428	90	135
3) 1) plus 2)	1,740	289	493

* The chick embryo enzyme contained 150 µg of protein per tube; the fibroblast preparations contained 200 µg protein per tube.

** WI-38 fibroblasts, passage 38 at confluency, were incubated for 3 hrs with or without(control) 0.1 mM L-ascorbic acid. Cells were harvested after trypsinization, washed and sonicated and the enzymic activity in the cell free preparation was assayed as described under "Methods".

haps cross-linked peptides found in material designated as the collagen "core". (The "core" constitutes about 10% of a typical collagen and consists of peptides arising from the "non-helical" terminal regions of α-chains and from the so-called "amorphous" regions ((cf ref. 2)). The possibility remains that the residual inhibition of hydroxylase by collagenase-digested collagen was due to peptides other than the groups just delineated.

Various collagens may be "underhydroxylated" in the sense that experimentally they are capable, in their heat-denatured forms, of undergoing further enzymic hydroxylation (7). The observed inhibition of hydroxylase by exogenous heat-denatured collagen might then in fact represent a dilution of radioactive substrate rather than a true inhibition. To

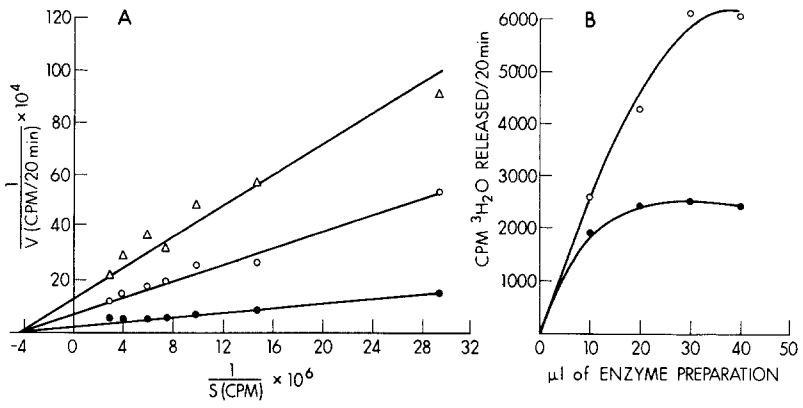

Fig. 1. Effect of calf skin collagen on chick embryo prolyl hydroxylase. The specific activity of the substrate was 2.2×10^6 cpm/mg. A: Double reciprocal plot in the presence and absence of calf skin collagen. ●——●, no inhibitor; o——o, 25 µg of calf skin collagen; Δ——Δ, 50 µg of calf skin collagen. B: o——o, chick embryo prolyl hydroxylase diluted 1:2 with water; ●——● enzyme diluted 1:2 with a solution containing 0.83 mg/ml of calf skin collagen.

examine this possibility, ^{3}H-labeled collagen was tested both as substrate and inhibitor for the hydroxylase of chick embryos and of WI-38 fibroblasts. Table II shows that ^{3}H-labeled exogenous collagen, in its heat-denatured form, was in itself an extremely poor substrate for hydroxylase; yet it inhibited the hydroxylation of protocollagen at least 70%.

The substrate specificity and inhibition of prolyl hydroxylase preparations have been studied using various peptides (8-11), reduced-carboxymethylated ascaris cuticular collagen (12), and protocollagen (13). Certain polytripeptides (9,10) and poly-L-proline (13) competitively inhibited prolyl hydroxylase activity. Bradykinin and some of its analogs can be hydroxylated in the proline residue at position 3 (11). Yet bradykinin substituted in the same position by a structural analog of proline was a competitive inhibitor for hydroxylation of reduced-carboxymethylated ascaris collagen (12). Figure 1 shows the inhibition of chick embryo prolyl hydroxylase by thermally denatured calf skin collagen. This inhibition was non-competitive (Fig. 1A); and K_I was lower than K_M by at least an order of magnitude (0.16 µM compared with 2.5 µM). Non-competitive inhibition of

TABLE III

Inhibition of prolyl hydroxylase from WI-38 fibroblasts by endogenous and exogenous heat-denatured collagens

Additions	cpm of ^3H_2O released	% inhibition
1) None*	2,913	-
2) Heat-denatured enzyme preparation (0.6 mg protein)**	749	74
3) As for 2) after collagenase treatment***	1,913	34
4) Ichthyocol, 128 µg	255	91
5) As for 4) after collagenase treatment***	1,864	36

* Confluent WI-38 fibroblasts, passage 23, served as the source of enzyme in this experiment. 150 µg protein of enzyme preparation was added to each tube. Other details are described under "Methods".

** Heat-inactivation was performed by immersion in a boiling water bath for 10 min. The boiled preparation had no detectable enzymatic activity.

*** Digested with collagenase as described in Table I.

chick embryo and WI-38 fibroblast prolyl hydroxylase was also observed with other collagens (unpublished results).

Since crude prolyl hydroxylase preparations contain small and perhaps variable amounts of collagen, the measured hydroxylase activity may not always reflect the true enzyme content. Thus, Fig. 1B demonstrates that dilution of chick embryo hydroxylase with heat-denatured exogenous collagen (from calf skin) caused considerable decrease in both rate and degree of hydroxylation of protocollagen. This pattern is typical for preparations of other enzymes containing a constant amount of inhibitor (14). Table III shows that hydroxylase activity of WI-38 fibroblasts was also strongly inhibited by addition of

heat-denatured ichthyocol or by addition of heat-inactivated hydroxylase preparation per se. In either case, pre-treatment of the inhibitor with collagenase considerably obviated the inhibition.

The occurrence of macromolecular substrates in close association with enzymes acting in their biosynthesis, modification or degradation is not rare; and indeed Peterkofsky and Udenfriend (15) presented evidence for occurrence of hydroxylase, protocollagen and collagen in microsomes isolated from chick embryos. Results presented here suggest that prolyl hydroxylase could be regulated biologically by the concentration or amount of hydroxylated α-chains or pro-α-chains; these may bind the enzyme strongly. A corollary could be that maximum hydroxylase activity obtains when such hydroxylated chains are removed from the site of enzymic action or transported out of the cell. A further consequence of this mode of regulation is that hydroxylation might be prevented by promoting intracellular accumulation of hydroxylated collagen chains. One should note that lysyl hydroxylase activity is also strongly inhibited by various collagens (B. Oppenheim and S. Englard, manuscript in preparation).

Finally, a few words should be made about "underhydroxylation" of collagen that has been considered a normal biological variable (16). Since prolyl hydroxylase acts on single chains, primarily as they are being synthesized on polyribosomes, evidence for "underhydroxylation" cannot be adduced from experiments in which triple-stranded collagen is extracted from a tissue, denatured with separation of chains, and then allowed to be further hydroxylated by an enzyme preparation. Under such circumstances an artificial system has been created (removal of polyribosomes, presence of complete chains devoid, however, of the portions cleaved by procollagen peptidase, etc.), so that additional hydroxylation may represent the kind observed with synthetic polypeptides or with various bradykinins. In such experiments controls for the sites and rates of hydroxylation are different from those in the biological situation. However, if one indeed analyzes a highly purified collagen with no other heterogeneity, the finding of identical sequences

except for differing degrees of hydroxylation of prolyl residues would constitute evidence for biological variability of hydroxylation.

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