Department of Biochemistry,
University of California
Riverside, Calif. 92502 (U.S.A.)

R. J. L. BONDAR*
N. G. PON

1 A. TIETZ AND S. OCHOA, *Arch. Biochem. Biophys.*, 78 (1958) 477.
2 A. M. REYNARD, L. F. HASS, D. D. JACOBSEN AND P. D. BOYER, *J. Biol. Chem.*, 236 (1961) 2277.
3 G. L. COTTAM, F. P. KUPIECKI AND M. J. COON, *J. Biol. Chem.*, 243 (1968) 1630.
4 *Sephadex Ion Exchangers, An Outstanding Aid in Biochemistry*, Pharmacia, Uppsala.
5 O. LINDBERG AND L. ERNSTER, in D. GLICK, *Methods of Biochemical Analysis*, Vol. 3, Interscience, New York, 1956, p. 1ff.
6 B. J. DAVIS, *Ann. N.Y. Acad. Sci.*, 121 (1964) 404.
7 D. E. WILLIAMS AND R. A. REISFELD, *Ann. N.Y. Acad. Sci.*, 121 (1964) 373.
8 T. BÜCHER AND G. PFLEIDERER, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. 1, Academic Press, New York, 1955, p. 435ff.
9 N. G. PON AND R. J. L. BONDAR, *Anal. Biochem.*, 19 (1967) 272.
10 F. NOLL, W. NEUMANN AND C. LITTMANN, *Acta Biol. Med. Ger.*, 16 (1965) 115.
11 F. P. KUPIECKI AND M. J. COON, *J. Biol. Chem.*, 235 (1960) 1944.
12 G. BEISENHERZ, T. BÜCHER AND K-H. GARBADE, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. 1, Academic Press, New York, 1955, p. 391.
13 O. WARBURG AND W. CHRISTIAN, *Biochem. Z.*, 310 (1941) 384.
14 J. B. MELCHIOR, *Biochemistry*, 4 (1965) 1518.

Received July 21st, 1969

* Present address and to whom all inquiries should be addressed: Department of Biochemistry, Michigan State University, East Lansing, Mich. 48823, U.S.A.

BBA 63434

Hydroxylation of proline and lysine in protocollagen involves two separate enzymatic sites

The hydroxyproline and hydroxylysine in collagen are synthesized by the hydroxylation of proline[1] and lysine[1,2] after these amino acids have been incorporated into a polypeptide precursor of collagen called protocollagen[1]. Most of the enzymatic activity for the hydroxylation of both the proline and lysine in protocollagen is found in the 150 000 × *g* supernatant fraction of chick embryo homogenates[3], and the cofactor or cosubstrate requirements for both hydroxylations are O_2, Fe^{2+}, α-ketoglutarate, and probably L-ascorbate[1–6]. It therefore seemed possible that the same enzyme is involved in both hydroxylations[3,5]. Results to be reported in this paper indicate that the two hydroxylations require separate enzymatic sites and probably separate proteins.

[^{14}C]Proline-labeled and [^{14}C]lysine-labeled protocollagen were prepared from chick embryo cartilage incubated with α,α′-dipyridyl and L-[$^{14}C_5$]proline (New England Nuclear Corp.), 209 μC/μmole, or L-[$^{14}C_6$]lysine (New England Nuclear Corp.), 248 μC/μmole[7]. The enzymatic reactions for the synthesis of [^{14}C]hydroxyproline and [^{14}C]hydroxylysine were carried out for 1 h at 37° in a final volume of 4 ml which

contained protocollagen substrate, 0.01–12 mg of enzyme protein, 0.05 mM $FeSO_4$, 0.5 mM α-ketoglutarate, 2 mM L-ascorbate (Sigma), 0.1 mg/ml catalase (Calbiochem), and 0.05 M Tris–HCl buffer adjusted to pH 7.8 at 25° (ref. 6). The incubations with [^{14}C]lysine-labeled protocollagen were stopped by the addition of 40 ml of acetone in the cold. The precipitated protein was centrifuged down at 1500 $\times g$, dissolved in 1.5 ml water, and [^{14}C]hydroxylysine was assayed by periodate oxidation[8]. The total ^{14}C content of each sample was determined by counting an aliquot of the original [^{14}C]lysine-labeled protocollagen preparation. Samples incubated with [^{14}C]proline-labeled protocollagen were assayed as described previously[3,5].

Since preparation of large quantities of ^{14}C-labeled protocollagen is cumbersome and costly, limiting quantities of substrate were used in the enzymatic reactions. When [^{14}C]lysine-labeled protocollagen was used as a substrate, the extent of hydroxylation of the substrate was proportional to enzyme concentrations over a significant range (Fig. 1B). Excess amounts of enzyme were required to give maximal hydroxylation of the substrate. The same relationship between enzyme concentration and the extent of hydroxylation was previously found for the hydroxylation of proline in [^{14}C]proline-labeled protocollagen under similar conditions[3,10] (Fig. 1A). Several concentrations of each enzyme fraction were examined in order to determine the amount of enzyme that still gave a linear relation between percent hydroxylation and enzyme concentration. Because of variations among different preparations of protocollagen[3], each experiment reported here was carried out with aliquots of the same protocollagen preparation which had been stored for less than 3 weeks.

Further examination of the specificity of the cofactor and cosubstrate requirements for the hydroxylation of lysine in protocollagen[2,5] revealed that as with the

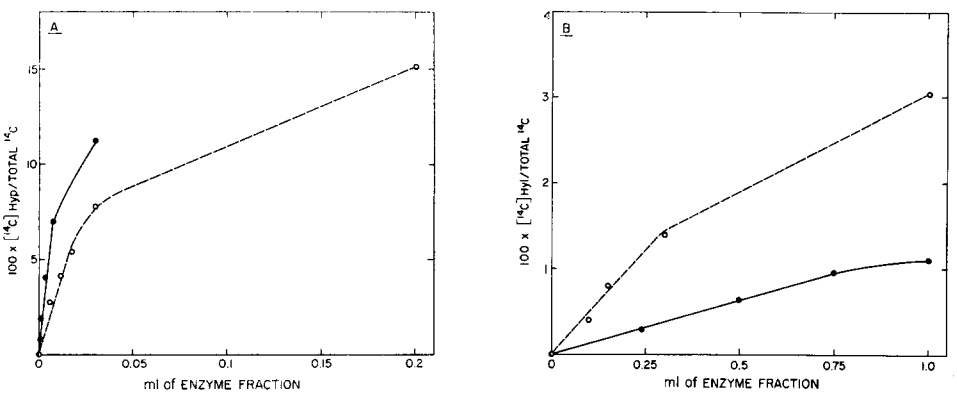

Fig. 1. A. Hydroxylation of proline by a crude extract and by a partially purified fraction of protocollagen proline hydroxylase from chick embryos. The extract was prepared by homogenizing twenty 12-day-old chick embryos in 85 ml 0.01 M KCl–0.05 M Tris–HCl, pH 7.8, at 4°, and centrifuging the homogenate at 150 000 $\times g$ for 1.5 h. The purified enzyme was the calcium phosphate gel fraction[9] which was dialyzed overnight against the KCl–Tris buffer before use. Assays with synthetic substrates indicated that the specific activity of the purified fraction was over 20 times that of the extract[9]. The substrate was [^{14}C]proline-labeled protocollagen (60 000 disint./min). ○ — — — ○, crude extract; ●—●, purified fraction. B. Hydroxylation of lysine by a crude extract and by a partially purified fraction of protocollagen proline hydroxylase. Conditions and curves as in A except that the substrate was [^{14}C]lysine-labeled protocollagen (300 000 disint./min).

hydroxylation of proline[4,6], α-ketoglutarate, L-ascorbate and iron were the most active cofactors or cosubstrates with crude preparations of enzyme. No synthesis of [^{14}C]hydroxylysine was observed, under the conditions described in Fig. 1, when α-ketoglutarate was replaced by equimolar concentrations of citrate, *cis*-aconitate, isocitrate, succinate, fumarate, L-malate, glutarate, malonate and α-ketobutyrate. Replacement of the 0.5 mM α-ketoglutarate with equimolar concentration of pyruvate, oxaloacetate, L-glutamate, ketomalonate, and dihydroxymaleate resulted in the synthesis of less than 40% of the [^{14}C]hydroxylysine synthesized in the presence of α-ketoglutarate. The requirement for L-ascorbate could not be replaced by 2 mM NADH and NADPH. With 2 mM (2-amino, 6,7-dimethyl, 4-hydroxy, 5,6,7,8)-tetrahydropteridine, tetrahydrofolic acid, and dihydroxymaleic acid the amount of [^{14}C]-hydroxylysine synthesized was less than 40% of that synthesized with equimolar amounts of L-ascorbate. When the L-ascorbate was replaced with equimolar D-isoascorbate, the amount of [^{14}C]hydroxylysine synthesized was 80% to 90% of that synthesized in the control. No activity was found when Fe^{2+} was replaced by equimolar Cu^{2+}, Mn^{2+}, Mg^{2+}, Ca^{2+}, Zn^{2+}, Cd^{2+}, Ni^{2+} and Co^{2+}. However, 0.05 mM Fe^{3+} was equally as effective as 0.05 mM Fe^{2+}, probably because Fe^{3+} is reduced to Fe^{2+} by the ascorbate in the enzymatic system.

Previous studies demonstrated that poly-L-proline in the form II conformation is a competitive inhibitor of protocollagen proline hydroxylase[6], and that denatured collagen from the cuticle of *Ascaris lumbricoides*[11] and synthetic polymers of the structure $(Gly–Pro–Pro)_n$[6] are substrates for the enzyme. When any of these three polypeptides were added to an enzymatic system containing both hydroxylases, the

TABLE I

EFFECT OF POLY-L-PROLINE, $(Gly–Pro–Pro)_n$, AND REDUCED AND CARBOXYMETHYLATED ASCARIS COLLAGEN ON THE HYDROXYLATION OF [^{14}C]PROLINE- AND [^{14}C]LYSINE-LABELED PROTOCOLLAGEN

[^{14}C]Proline-labeled protocollagen (100 000 disint./min) and [^{14}C]lysine-labeled protocollagen (300 000 disint./min) were incubated with 0.31 mg and 0.96 mg[13], respectively, of a 0–50% $(NH_4)_2SO_4$ fraction of enzyme as described in the text. The 0–50% $(NH_4)_2SO_4$ fraction was prepared as described previously[6] except that the homogenate was stirred for 2 h with 0.1% Triton X-100 (Packard Instruments), and nucleic acids were precipitated with 1.5 mg of streptomycin sulfate per mg nucleic acid before the fractionation with $(NH_4)_2SO_4$ was carried out.

Addition	*Concn. (μg/ml)*	*Proline hydroxylase*		*Lysine hydroxylase*	
		$100 \times \frac{[^{14}C]\,Hyp}{total\ ^{14}C}$	*% of control*	$100 \times \frac{[^{14}C]\,Hyl}{total\ ^{14}C}$	*% of control*
None	—	13.25	100	2.68	100
Poly-L-proline*	10	1.18	9	3.13	117
	100	0.47	4	2.78	104
$(Gly–Pro–Pro)_n$**	10	10.91	82	2.49	93
	100	4.09	31	2.75	103
RCM collagen***	10	0.31	2	3.14	117
	100	0.07	1	2.85	106

* Poly-L-proline in the form II conformation and with average molecular weight of 15 000.
** Polymer with average molecular weight of about 4000.
*** Reduced and carboxymethylated collagen from the cuticle of *Ascaris lumbricoides*. Denatured by boiling for 10 min[11].

polypeptides inhibited the hydroxylation of [^{14}C]proline in protocollagen but did not inhibit the hydroxylation of [^{14}C]lysine (Table I).

In further experiments, the relative amounts of lysine and proline hydroxylase were measured in crude extracts of chick embryos and in a partially purified fraction of proline hydroxylase from the same source. The enzymatic activities were compared by estimating the amount of enzyme required to obtain 1% hydroxylation of [^{14}C]-lysine-labeled protocollagen and 4% hydroxylation of [^{14}C]proline-labeled protocollagen. On this basis the ratio of lysine hydroxylase to proline hydroxylase decreased by a factor of 10 when the extract was compared to the partially purified fraction (Fig. 1). Also, recent experiments by K. I. KIVIRIKKO (personal communication) show that very highly purified proline hydroxylase fractions contain no lysine hydroxylase activity.

The observation that competing polypeptides inhibit the hydroxylation of proline without inhibiting the hydroxylation of lysine indicates that the hydroxylations of lysine and proline in protocollagen involve two separate enzymatic sites. The loss of lysine hydroxylating activity upon purification of protocollagen proline hydroxylase suggests that the two enzymatic activities are contained in two separate proteins. However, the procedures used to purify the proline hydroxylase may have selectively destroyed lysine hydroxylating activity in the same protein.

This work was supported in part by research grants FR-107, AM-5459, and HD-183 from the National Institutes of Health, U.S. Public Health Service.

Departments of Biochemistry and Medicine,
University of Pennsylvania,
and the Philadelphia General Hospital,
Philadelphia, Pa. (U.S.A.)

ELSA WEINSTEIN
NELLY BLUMENKRANTZ
DARWIN J. PROCKOP

1 J. ROSENBLOOM AND D. J. PROCKOP, in J. E. DUNPHY AND W. VAN WINKLE, JR., *Repair and Regeneration: The Scientific Basis for Surgical Practice*, McGraw-Hill, New York, 1969, p. 117.
2 E. HAUSMANN, *Biochim. Biophys. Acta*, 133 (1967) 591.
3 K. I. KIVIRIKKO AND D. J. PROCKOP, *Arch. Biochem. Biophys.*, 118 (1967) 616.
4 J. J. HUTTON, JR., A. L. TAPPEL AND S. UDENFRIEND, *Arch. Biochem. Biophys.*, 118 (1967) 231.
5 K. I. KIVIRIKKO AND D. J. PROCKOP, *Proc. Natl. Acad. Sci. U.S.*, 57 (1967) 782.
6 K. I. KIVIRIKKO AND D. J. PROCKOP, *J. Biol. Chem.*, 242 (1967) 4007.
7 T. TAKEUCHI AND D. J. PROCKOP, *Gastroenterology*, 56 (1969) 744.
8 N. BLUMENKRANTZ AND D. J. PROCKOP, *Anal. Biochem.*, in the press.
9 J. HALME AND K. I. KIVIRIKKO, *Federation European Biochem. Soc. Letters*, 1 (1968) 223.
10 J. J. HUTTON, JR., A. L. TAPPEL AND S. UDENFRIEND, *Anal. Biochem.*, 16 (1966) 384.
11 D. FUJIMOTO AND D. J. PROCKOP, *J. Biol. Chem.*, 243 (1968) 4138.
12 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.

Received August 4th, 1969

Biochim. Biophys. Acta, 191 (1969) 747–750