LOCALIZATION OF THE SITE OF PROLINE HYDROXYLATION DURING THE CELL-FREE BIOSYNTHESIS OF COLLAGEN

Beverly Peterkofsky and Sidney Udenfriend

Laboratory of Clinical Biochemistry, National Heart Institute, Bethesda, Maryland

Received June 20, 1963

We have previously reported a cell-free system derived from chick embryo homogenates in which proline-C^{14} is incorporated into protein-bound hydroxyproline associated with the microsomal fraction (Peterkofsky and Udenfriend, 1961). No significant amounts of protein-bound hydroxyproline-C^{14} were found in the soluble protein fraction. The present communication describes studies carried out with this system which give some information concerning the site of proline hydroxylation.

Materials and Methods

The system used was essentially the same as that described previously except that the 15,000 x g supernatant fraction, used as the enzyme extract, was dialyzed against three changes of 0.25 M sucrose (2 liters each), over a period of 1-1/2 hours at 4°. The isolation of the hydroxyproline-containing protein by hot TCA[1] extraction was also described in the previous report. Radioactivity of protein-bound proline and hydroxyproline was determined after hydrolysis by the method of Peterkofsky and Prockop (1962). Incubation conditions were slightly modified so that the incubation mixture contained the following components, in μmoles: Tris·HCl, pH 7.6, 500; ATP, 20; creatine phosphate, 200; $MgCl_2$, 40; KCl, 200; sucrose, 2500. L-Proline-C^{14}, uniformly labeled, was added as indicated under the various tables and approximately 50 to 100 mg of dialyzed S-15 fraction was added.

[1] Abbreviations used: TCA, trichloroacetic acid; ATP, adenosine triphosphate; S-RNA, soluble-ribonucleic acid; RNAase, ribonuclease; S-15 fraction, 15,000 x g supernatant.

The final volume was 10 ml and incubations were carried out in air in 50 ml Erlenmeyer flasks for 2 hours at 37° with shaking. Further details of the properties of this system and experiments showing the similarity of the hydroxyproline-containing microsomal protein to collagen have been submitted for publication (Peterkofsky and Udenfriend, 1963).

Results and Discussion

When the time course of proline-C^{14} incorporation into protein-bound proline and hydroxyproline was studied previously with the undialyzed extract, a lag phase of 10 to 15 minutes occurred before radioactivity was detected in protein-bound hydroxyproline. Using dialyzed extracts, this lag phase has been extended to approximately 30 minutes (Fig. 1). When puro-

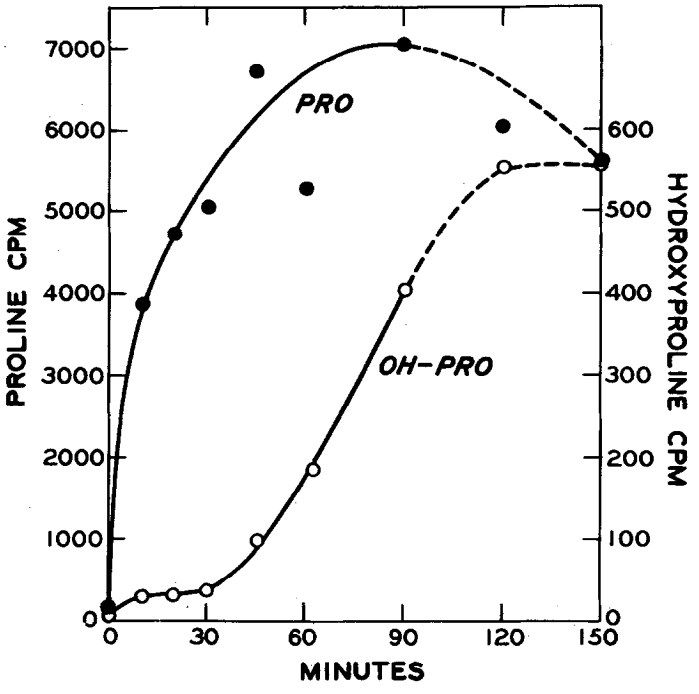

Figure 1. Time course of incorporation of proline-C^{14} into protein-bound imino acids in the hot TCA extractable, nondialysable, fraction of microsomes. The results are the average of two overlapping experiments. The dotted line curves represent portions of the second experiment which have been extrapolated to coincide with the values obtained for the first experiment since, although the two curves were parallel, the absolute values differed by about one-half.

mycin, which inhibits the transfer of amino acids from S-RNA to microsomal-bound polypeptide (Yarmolinsky and de la Haba, 1959; Nathans, et al., 1962; Arlinghaus, et al., 1963), was added at the beginning of the incubation, both activities were almost completely inhibited (Table I, line 2). However, when puromycin was added after 30 minutes, at the end of the lag phase, inhibition of both activities was no longer significant (Table I, lines 3 and 5). This was not unexpected for proline-C^{14} incorporation into protein-

TABLE I

The Effects of Puromycin and Ribonuclease on the Incorporation of Proline-C^{14} into Protein-bound Imino Acids

Experiment	Additions	Incorporation Into Proline		Incorporation Into Hydroxyproline	
		cpm	Percent Inhibition	cpm	Percent Inhibition
1	Control	2,055	0	209	0
	+ puromycin, 0.23 μmoles/ml				
	at 0 time	141	93	18	91
	at 30 mins.	1,505	27	169	19
2	Control	11,253	0	1,058	0
	+ puromycin, 0.28 μmoles/ml				
	at 30 mins.	11,880	0	1,063	0
3	Control	16,110	0	1,481	0
	+ RNAase, 10 γ/ml				
	at 0 time	165	99	15	99
	at 20 mins.	10,610	34	194	87
	at 30 mins.	13,510	16	996	33
	at 110 mins.	15,630	3	1,366	8

The final proline specific activities in the various experiments were as follows, in cpm/mμmole: 1) 0.50×10^4, 2) 0.54×10^4, 3) 1.13×10^4 and approximately 1.0×10^7 cpm were added in each experiment. Crystalline pancreatic ribonuclease (Mann Research Corporation) was used and the puromycin was from Lederle Laboratories.

bound proline, since approximately 80% of its maximal incorporation had already occurred by 30 minutes (see Fig. 1). However, the marked decrease in inhibition of proline-C^{14} conversion to protein-bound hydroxyproline-C^{14} was rather unexpected since radioactivity is barely detectable in this substance at 30 minutes (see Fig. 1). These results suggest that the immediate precursor of protein-bound hydroxyproline had already been transferred to the microsomes by the end of the lag phase. This suggestion is further corroborated by experiments with ribonuclease added at varying time intervals. When added at zero time, ribonuclease caused almost complete inhibition of incorporation into both protein-bound imino acids, showing that inhibition occurred almost immediately (Table I, line 7). When ribonuclease was added towards the end of the lag phase, at 20 minutes, there was a reduction in the inhibition of proline-C^{14} incorporation into protein-bound proline corresponding to the extent of incorporation which had already occurred at this point (approximately 70% of the maximum incorporation occurs by 20 minutes, as can be calculated from Fig. 1). There was still a substantial inhibition of proline-C^{14} incorporation into protein-bound hydroxyproline, as one might expect. Upon addition at 30 minutes, however, inhibition was markedly reduced from 87% to 33%. Again, it should be pointed out that at 30 minutes there is as yet an insignificant amount of labeled protein-bound hydroxyproline produced. This suggests that toward the end of the lag phase, the precursor of protein-bound hydroxyproline is converted to a form relatively resistant to ribonuclease attack.

Since the hydroxyl oxygen of hydroxyproline is derived from molecular oxygen (Prockop, et al., 1962; Fujimoto and Tomiya, 1962), it was possible to inhibit hydroxylation by incubating under anaerobic conditions. When incubations of the cell-free system were carried out under nitrogen for the entire 2-hour period, there was little effect on the incorporation into protein-bound proline, but incorporation into protein-bound hydroxyproline

was appreciably diminished (Table II, lines 3 and 4). It can be seen, however, that the requirement for oxygen occurred after the 30 minute lag phase, since anaerobiosis during the last 90 minutes produced the same inhibition as anaerobiosis for the entire 2 hours, while anaerobiosis during the lag phase only had no effect (Table II, lines 5 and 6).

TABLE II

The Effect of Anaerobiosis on the Incorporation of Proline-C^{14} into Protein-Bound Imino Acids

Gas Phase During Incubation			Incorporation into Protein-Bound			
			Proline		Hydroxyproline	
0 - 30 mins.	30 - 120 mins.	Expt. No.	cpm	Percent Inhibition	cpm	Percent Inhibition
Air	Air	1	6,040	0	473	0
		2	11,253	0	1,058	0
N_2	N_2	1	5,490	9	221	53
		2	8,900	21	509	52
Air	N_2	2	10,610	6	473	55
N_2	Air	2	11,060	2	1,058	0

Experiment No. 1 was carried out in large Thunberg tubes containing 5 ml of incubation mixture in each; two tubes were combined for analysis. The results for each condition are the average of two such analyses. Experiment No. 2 was carried out in 50 ml Erlenmeyer flasks designed to allow evacuation and incubation in a manner similar to using Thunberg tubes. All vessels were alternately evacuated and flushed several times with prepurified N_2 containing less than 0.002% of O_2 (Southern Oxygen Company) and finally either left closed under N_2 or open, as indicated. The final specific activities of the free proline-C^{14} were, in cpm/mμmole: expt. No. 1, 0.54×10^4; expt. No. 2, 1.18×10^4. Approximately 1.0×10^7 cpm were added in each expt.

Since proline, but not free hydroxyproline, is incorporated into collagen (Stetten, 1949), it is apparent that during the sequence of reactions leading to the biosynthesis of this protein, a proline intermediate is hydroxylated. The lag phase described in these experiments is evidently a period during which the proline derivative to be hydroxylated is produced and transferred to the microsomes. Since almost all the hydroxyproline-containing protein was found to be associated with the microsomes, it would suggest that the last stage in protein synthesis, release of soluble protein (Morris and Schweet, 1961), is not functioning in this system. Therefore the substrate for hydroxylation would appear to be a microsomal RNA-bound polypeptide of considerable size. These findings would rule out an S-RNA-hydroxyproline intermediate, as suggested by Manner and Gould (1962).

References

Arlinghaus, R., Favelukes, G. and Schweet, R., Biochem. Biophys. Res. Comm., 11, 92 (1963).
Fujimoto, D. and Tamiya, N., Biochem. J., 84, 333 (1962).
Manner, G. and Gould, B. S., Fed. Proc. 21, 169 (1962).
Morris, A. and Schweet, R., Biochim. Biophys. Acta, 47, 415 (1961).
Nathans, D., Von Ehrenstein, G., Monro, R. and Lipmann, F., Fed. Proc., 21, 127 (1962).
Peterkofsky, B. and Udenfriend, S., Biochem. Biophys. Res. Comm., 6, 184 (1961).
Peterkofsky, B. and Prockop, D. J., Anal. Biochem., 4, 400 (1962).
Peterkofsky, B. and Udenfriend, S., submitted to J. Biol. Chem., (1963).
Prockop, D. J., Kaplan, A. and Udenfriend, S., Biochem. Biophys. Res. Comm., 9, 162 (1962).
Stetten, M. R., J. Biol. Chem., 181, 31 (1949).
Yarmolinsky, M. B. and de la Haba, G. L., Proc. U. S. Nat'l. Acad. Sci., 45, 1721 (1959).