SECRETION OF UNHYDROXYLATED CHICK TENDON PROCOLLAGEN

Sergio A. Jimenez* and Ronald Yankowski

Department of Medicine, University of Pennsylvania School of Medicine and Philadelphia General Hospital

Philadelphia, Pa. 19104

Received September 23,1975

SUMMARY: The secretion of unhydroxylated procollagen at 37° by isolated chick tendon fibroblasts independent of protein synthesis was examined. The data showed that intact molecules were secreted and that their degradation was an extracellular event. The kinetics of secretion indicated that most of the secreted procollagen appeared in the medium during the initial 30 min following inhibition of protein synthesis and only an additional 35% reached the extracellular space in the subsequent 90 min. The pattern of secretion suggested the existence of an intracellular binding site for the unhydroxylated molecules which was saturated during the early period of secretion. It is speculated that such a binding site could be the enzyme prolyl hydroxylase which has a high affinity for unhydroxylated procollagen at 37°.

INTRODUCTION

One of the crucial steps during collagen biosynthesis is the hydroxylation of appropiate prolyl and lysyl residues while nascent chains are being assembled on the ribosomes. When the enzymatic hydroxylation of prolyl and lysyl residues was inhibited, unhydroxylated procollagen was synthesized but it was not secreted at a normal rate and accumulated intracellularly (1-4). Characterization of the secreted proteins indicated that only small molecular weight peptides were present and it was suggested that only degradation products were secreted by the inhibited cells (3-5). Recent studies have shown that hydroxyproline is crucial for the thermal stability of collagen and that unhydroxylated molecules are not triple helical at normal body temperatures (6,7) and it has been suggested that a triple helical conformation was required for normal secretion. In the present

* To whom correspondence should be addressed.

study we have further examined the secretion of unhydroxylated procollagen under conditions at which the molecules were not triple helical.

MATERIALS AND METHODS

Fibroblasts were obtained from 17-day old chick embryo tendons as previously described (2). Subsequently 10^7 cells/ml were incubated at 37° in Krebs medium containing 2% fetal calf serum, 25 ug/ml ascorbic acid and 0.5mM α, α' dipyridyl. After 20 min preincubation ^{14}C proline was added and the incubation was continued for another 60 min. Further protein synthesis was then inhibited by addition of cycloheximide. The cells were separated from the media by centrifugation and then they were resuspended in fresh media containing cycloheximide and α, α' dipyridyl and the incubation was continued at 37°. Aliquots were obtained at the intervals indicated and were centrifuged at 1200 xg for 10 min to separate cells from the secreted proteins present in the media. Cells and media were prepared and chromatographed on SDS* - Agarose columns as reported previously (8). The ^{14}C-hydroxyproline content of the collagen samples labeled with ^{14}C proline was assayed by a specific chemical procedure (9). The radioactivity of the medium and intracellular proteins was determined directly after extensive dialysis against running tap water.

RESULTS

As shown previously, incubation of the isolated chick tendon fibroblasts with 0.5 mM α, α'- dipyridyl resulted in the synthesis and intracellular accumulation of procollagen containing no detectable amounts of ^{14}C-hydroxyproline. When the secretion of ^{14}C-labeled unhydroxylated procollagen at 37° was examined, it was evident that about 40% of the protein which had accumulated intracellularly was secreted in the 120 min following inhibition of protein synthesis. When the proteins present in the medium at various time intervals were chromatographed in SDS-Agarose columns, (Fig. 1A) it was found that at 30 min most of the radioactivity eluted in a position comparable to that of procollagen and no evidence for small molecular weight fragments was present. At 90 min however, the main peak appeared broader and some small molecular weight fragments were found. In the 180 min sample, essentially no radioactivity was found in the area of elution of procollagen and the majority of the labeled proteins appeared to have a molecular weight smaller than that of collagen alpha-chains; over 50% of the recovered radio-

* Abbreviation: SDS; sodium dodecyl sulfate

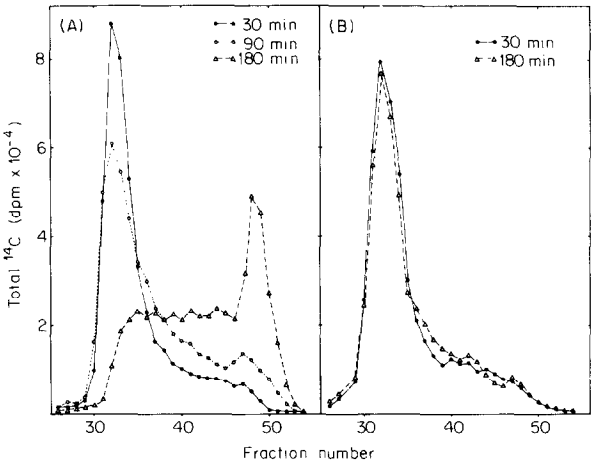

Fig. 1. SDS-Agarose gel chromatography of secreted and intracellular labeled proteins at various time-periods following inhibition of protein synthesis. Cells and medium were prepared for chromatography and aliquots containing approximately 200,000 dpm were chromatographed as described previously. Marker β rat skin collagen eluted at fraction 30 and α rat skin collagen eluted at fraction 35. A, Medium proteins; B, Intracellular proteins.

activity eluted near the end of the effluent volume. When the intracellular proteins were similarly examined, (Fig. 1B), the pattern of the chromatograms was identical at all periods, the major portion of the labeled protein eluting in the region of procollagen. No evidence to suggest intracellular degradation was found.

The kinetics of secretion of the labeled proteins (Fig. 2) showed an early phase at which secretion occurred at a rapid rate. At about 30 min the rate decreased markedly and in the subsequent 90 min the secretion was very slow. Approximately 65% of the secreted radioactivity appeared in the medium in the first 30 min and only an additional 35% of labeled proteins were secreted in the subsequent 90 min. The total amount of radioactivity recovered from the cells and medium, remained constant for the duration of the experiment.

DISCUSSION

Previous studies showed that tendon fibroblasts incubated in the presence of α, α' - dipyridyl or under anaerobic conditions (3-5) synthesized

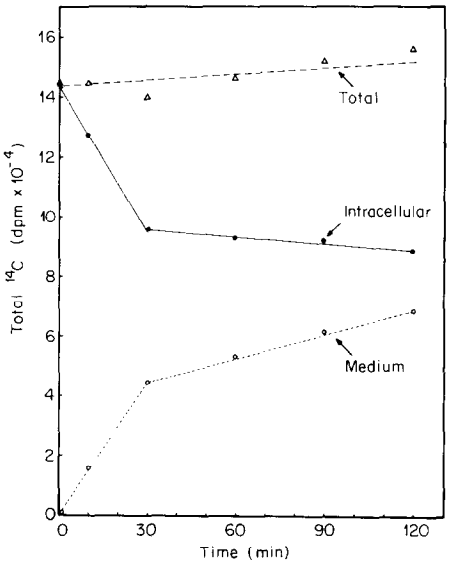

Fig. 2. Kinetics of secretion of unhydroxylated procollagen at 37°. Fibroblasts were incubated at 37° at a concentration of 10^7/ml in 10 ml of Krebs medium containing 0.5 mM α, α'- dipyridyl. After 20 min 5uCi/ml of ^{14}C proline was added and the incubation was continued for 60 min. Cycloheximide was then added to a final concentration of 100 ug/ml and the cells were separated from the media by centrifugation. The cells were resuspended in 10 ml of fresh Krebs medium containing 0.5 mM α, α'- dipyridyl and 100 ug/ml cycloheximide. After resuspension the incubation was continued at 37° and 1.5 ml aliquots were obtained at 10, 30, 60, 90 and 120 min after addition of cycloheximide. The aliquots were centrifuged, the cells were resuspended in distilled water, aggitated in a vortex and then dialyzed against running tap water. The media were directly dialyzed against running tap water. After dialysis aliquots were counted in a triton-toluene scintillant.

unhydroxylated procollagen. The unhydroxylated procollagen was apparently retained intracellularly and only small molecular weight products were present in the media. It was suggested that the unhydroxylated molecules were degraded intracellularly and that only degradation products were secreted. The results presented here indicate that unhydroxylated molecules which elute in a position comparable to that of procollagen on the SDS-Agarose columns, are present in the media of isolated tendon fibroblasts incubated at 37°. This result is similar to the findings of Ramaley et. al. in cultured fibroblasts (10). Furthermore, our results suggest that the degradation of these unhydroxylated molecules does not occur intra-

cellularly as suggested earlier, but it is an extracellular event. In the present study, the cells were washed and resuspended in fresh medium immediately after protein synthesis was inhibited. In the previous studies protein synthesis was not inhibited and the cells were incubated without additional washing and resuspension. It seems possible that in these studies, collagenase and protease activity remaining from the cell preparation or released by the cells themselves may have been responsible for the degradation of the secreted molecules.

The kinetics of secretion of the unhydroxylated molecules indicate that the secretory process occurs at two distinct rates. During the early period of secretion, the radioactive molecules are rapidly secreted in the medium and after 30 min the rate of secretion decreases significantly. This change in the rate of secretion could be consistent with the possibility that there is an intracellular binding site for the unhydroxylated procollagen which is saturated at the initial period of secretion and therefore permits the rapid transport of the excess unhydroxylated molecules to the extracellular space. When the equilibrium point is reached and the amount of intracellular procollagen equals that of the available binding sites, then the secretion rate diminishes markedly and becomes dependent on the affinity of the binding site for the unhydroxylated molecules. Previous studies on the kinetics of secretion of unhydroxylated collagen (4,11) are not directly comparable to the present study. In the previous reports protein synthesis was not inhibited, and the kinetic data are a composite of both synthesis and secretion. Also the possibility of degradation of the secreted molecules was not excluded.

The nature of the intracellular binding site can only be speculated. We suggest that prolyl hydroxylase may be the binding site since it has a very high affinity for non-triple helical procollagen (12-14) and it has been shown to form stable complexes with unhydroxylated substrates (15). Additional support for this possibility is apparent from experiments

which explored the secretion of unhydroxylated procollagen at a temperature below the denaturation temperature of the molecules (14,16). In these experiments the unhydroxylated procollagen was secreted at a faster rate when the temperature was maintained at 24°. Since the affinity of the prolyl hydroxylase markedly diminishes when the substrate is in a triple helical conformation, it is likely that the more rapid rate of secretion of unhydroxylated procollagen at 24° is due to the decreased affinity of the enzyme for the substrate. It can also be speculated that other enzymes involved in the intracellular post-ribosomal modifications of collagen may excert similar binding effects under circumstances where increased affinity of such enzymes for the collagen exist.

ACKNOWLEDGMENTS

We thank Dr. N. A. Kefalides for valuable criticism. We acknowledge the expert assistance of Mrs. P. Frontino and Mrs. C. Porter. Supported by the Philadelphia General Hospital Research Fund and the Barsumian Fund.

REFERENCES

1. JUVA, K., PROCKOP, D.J., COOPER., AND LASH, J. (1966) Science 152,92.
2. DEHM, P., AND PROCKOP, D.J. (1971) Biochim,Biophys. Acta 240,358.
3. RAMALEY, P.B. AND ROSENBLOOM, J. (1971) Fed. Eur. Biochem. Soc. Letters 15, 59.
4. JIMENEZ, S.A., DEHM, P., OLSEN, B.R., AND PROCKOP, D.J. (1973) J. Biol. Chem. 248,720.
5. UITTO, J., AND PROCKOP, D.J. (1974) Eur. J. Biochem. 43,221.
6. JIMENEZ, S.A., HARSCH, M., AND ROSENBLOOM, J. (1973) Biochem. Biophys. Res. Commun. 52, 106.
7. BERG, R., AND PROCKOP, D.J. (1973) Biochem. Biophys. Res. Commun. 52,115.
8. JIMENEZ, S.A., DEHM, P., AND PROCKOP, D.J. (1971) Fed. Eur. Biochem. Soc. Letters 17,245.
9. JUVA, K., AND PROCKOP, D.J. (1966) Anal. Biochem. 15, 77.
10. RAMALEY, P.B., JIMENEZ, S.A., AND ROSENBLOOM, J. (1973) Fed. Eur. Biochem. Soc. Lett. 33,187.
11. MARGOLIS, R.L., AND LUKENS, L.N. (1971) Arch. Biochem. Biophys. 147,612.
12. BERG, R.A., AND PROCKOP, D.J. (1973) Biochemistry 12,3395.
13. MURPHY, L., AND ROSENBLOOM, J. (1973) Biochem. J. 135,249.
14. JIMENEZ, S.A., HARSCH, M., MURPHY, L., AND ROSENBLOOM, J. (1974) J. Biol. Chem. 249,4480.
15. JUVA, K., AND PROCKOP, D.J. (1969) J. Biol. Chem. 244,6486.
16. JIMENEZ, S.A., AND YANKOWSKI, R. (1975) Fed. Proc. 34,563.