EVIDENCE FOR THE ASSOCIATION OF PROLYL HYDROXYLASE FROM CHICK EMBRYOS WITH MEMBRANES THROUGH MEMBRANE-BOUND PROCOLLAGEN

J. Farjanel, A. Chamson and J. Frey

Laboratoire de Biochimie, U.E.R. de Médecine,
42100 Saint-Etienne, France

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SUMMARY :

Proline hydroxylase of membrane pellets from tissues of chick embryo was solubilized by several extraction mediums. Three kinds of enzyme linkage with membranes were pointed out : a labile and non specific adsorption, a specific linkage on endogenous procollagen which is linked with the membranes, and a part of membranes themselves. Our results suggest that, in the liver, these three kinds of linkage exist. Whereas, in tibia bones only labile linkages appear by non specific adsorption or through the link with membrane procollagen.

INTRODUCTION :

For a long time, procollagen-prolyl-hydroxylase (P.P.H.) (EC 1.14.11.2) which catalyses the biosynthesis of hydroxyprolyl residues in collagen, was considered as a soluble enzyme located within cytosol (1, 2). Using differential centrifugation, Guzman et al. (3, 4), Harwood et al. (5) and Peterkofsky et al. (6) showed recently that prolyl hydroxylase was localized to a great extent within a rough microsomal fraction. By cytochemical techniques, Olsen et al. (7) and Al Adnani et al. (8) have shown that prolyl hydroxylase can be observed in the cisternae of endoplasmic reticulum. By subcellular fractionation, Guzman et al. (9) have also demonstrated the localization of both prolyl and lysyl hydroxylase within the cisternae of the rough endoplasmic reticulum.

In our laboratory, Helfre et al. (10, 11, 12) have characterized, by marker enzymes and electron microscopy, the subcellular fractions from chick embryo liver in which prolyl hydroxylase occurs, and they have pointed out that the highest specific activity of this enzyme was located in the endoplasmic reticulum.

Several mechanisms can account for the membrane localization :

- the enzyme is a part of the membrane structure ;
- the enzyme is linked within a stable enzyme - substrate complex, the substrate of which is bound with the endoplasmic membranes ;
- the enzyme is adsorbed on the membranes non specifically and to a greater or lesser degree.

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The present report concerns the verification of these hypotheses. For this purpose, Triton X 100 (0.1%) which does not alter prolyl hydroxylase activity was used to solubilize the membrane components and so release the major part of the prolyl hydroxylase activity. With the triton extract was compared that obtained by two other solubilizing medium :

- a tris-maleate buffer which can only disrupt without specificity a weakly linked fraction,
- a tris-maleate buffer including prolyl hydroxylase cofactors (Fe^{2+}, ascorbate, α-ketoglutarate) which start the enzymic hydroxylation of the prolyl residues and then break the membranous enzyme - substrate bond and make the enzyme soluble (indeed such a complex is stable only when the procollagen substrate is not hydroxylated (13)).

MATERIALS AND METHODS :

Prolyl hydroxylase assay :

Prolyl hydroxylase was measured by the method of Hutton et al. (14) as described in detail elsewhere (15,16). This method is based on the release of tritiated water into the incubation medium when a peptidyl (4-^{3}H) proline-rich, hydroxyproline deficient substrate prepared from 9 day old chick embryos is incubated with enzyme and cofactors aerobically. The labeled procollagen substrate was extracted three times by 0.5 N acetic acid, 12 hours each, and purification was performed with 1 M NaCl to obtain all the hydroxylable collagen in the supernatant.

Each assay mixture contained 0.5 ml of labeled substrate (approximately 100,000 dpm), 0.1 mM ferrous ammonium sulfate, 1 mM ascorbic acid, 0.5 mM α-ketoglutarate, 2 mg of catalase and amounts of enzyme found to be within the linear range of activity. The mixture was made up to a total volume of 2 ml with 25 mM tris - 50 mM maleate - 50 mM NaCl buffer (pH 7.1) in a 50 ml glass stoppered flask. After incubation aerobically at 38°C for 10 to 40 min the reaction was terminated by addition of 0.2 ml of 50 % trichloracetic acid. In the same way, a control is performed in the absence of enzyme.

The samples were stored at -25°C before measurement. The resulting tritiated water formed was collected by vacuum distillation, and 1 ml was counted with 13 ml of Bray's scintillation liquid. Activity is expressed as disintegration per min of tritiated water released in 20 min corrected for radioactivity released in the absence of enzyme.

The amount of protein in the enzyme extracts was determined by the method of Lowry.

Subcellular fractionation of cells :

We used 100 to 200 16 day old chick embryos as we have verified (10) that their liver contains the highest prolyl hydroxylase activity. The results in table I concern approximately 200 embryos for tibia extract and 800 embryos for liver extract.

The fractionation procedures are described in figure 1. Two kinds of buffers with and without $MgCl_2$, KCl and sucrose were used in order to preserve or not the integrity of the membrane bound polysomes (2 ml/g of tissue).

Solubilization of membrane bound prolyl hydroxylase :

The 200,000 x g pellet was gently resuspended in buffer and divided into three identical parts.

One part was preincubated for 30 min and then treated with Triton X 100 (0.1 % final concentration) immediatly before centrifugation at 200,000 x g for 1 hour. The supernatant contains the prolyl hydroxylase solubilized by the buffer and Triton at the same time. It was verified that Triton at this concentration does not alter prolyl hydroxylase activity.

A second part was also preincubated in the same manner, but Triton was not added before centrifugation. The supernatant contains the prolyl hydroxylase solubilized by the buffer only.

A third part was preincubated in the same manner, but the cofactors of the prolyl hydroxylase were added in the buffer. The cofactor mixture containing 0.1 mM ferrous ammonium sulfate, 1 mM ascorbic acid and 0.5 mM α-ketoglutarate, starts the enzyme activity and can break an enzyme - substrate complex if it exists in the membrane extract. Catalase (2 mg) was added in order to protect the enzyme from the effects of free radicals induced by the Fe^{2+} - ascorbate mixture. After centrifugation, the supernatant contains the prolyl hydroxylase solubilized by the buffer and released from an enzyme - substrate complex in which the substrate would be bound with the membranes.

RESULTS :

Table I lists the results from extraction with or without $Mg\ Cl_2$, KCl and sucrose which can prevent polysome disruption. The measured values in each solubilized fraction were multiplied by three to take into account the subdivision of the 200,000 x g pellet into three identical parts. These values could be compared to those obtained from every other fraction especially cytosol.

DISCUSSION :

Before discussing the mechanisms of prolyl hydroxylase solubilization it should be noted that measured activities were minimal values due to the necessary hard conditions of homogenization and preincubation : if a longer preincubation is used at a higher temperature than those described in figure 1, the mixture of buffer with cofactors solubilizes less enzyme activity than the single buffer. Likewise, when the catalase concentration, which prevents formation of free radicals, was not sufficient, we observed an increase of the extracted proteins and a decrease of the enzyme activity. In addition, when homogenization was carried out for a longer time than indicated in figure 1, the major part of the prolyl hydroxylase was recovered in the cytosol, especially in the case of tibia which was treated with Turax.

In the case of the extraction with a single buffer, we have verified that there was in the enzymic fraction no collagenase activity which could release prolyl hydroxylase from a membrane bound procollagen (17). Therefore, by comparing the results to those obtained with the mixture buffer - Triton X 100 (table II), several kinds of enzyme bond can be distinguished with membranes contained in a 200,000 x g pellet :

- the labile adsorption of the enzyme within the membrane fraction could

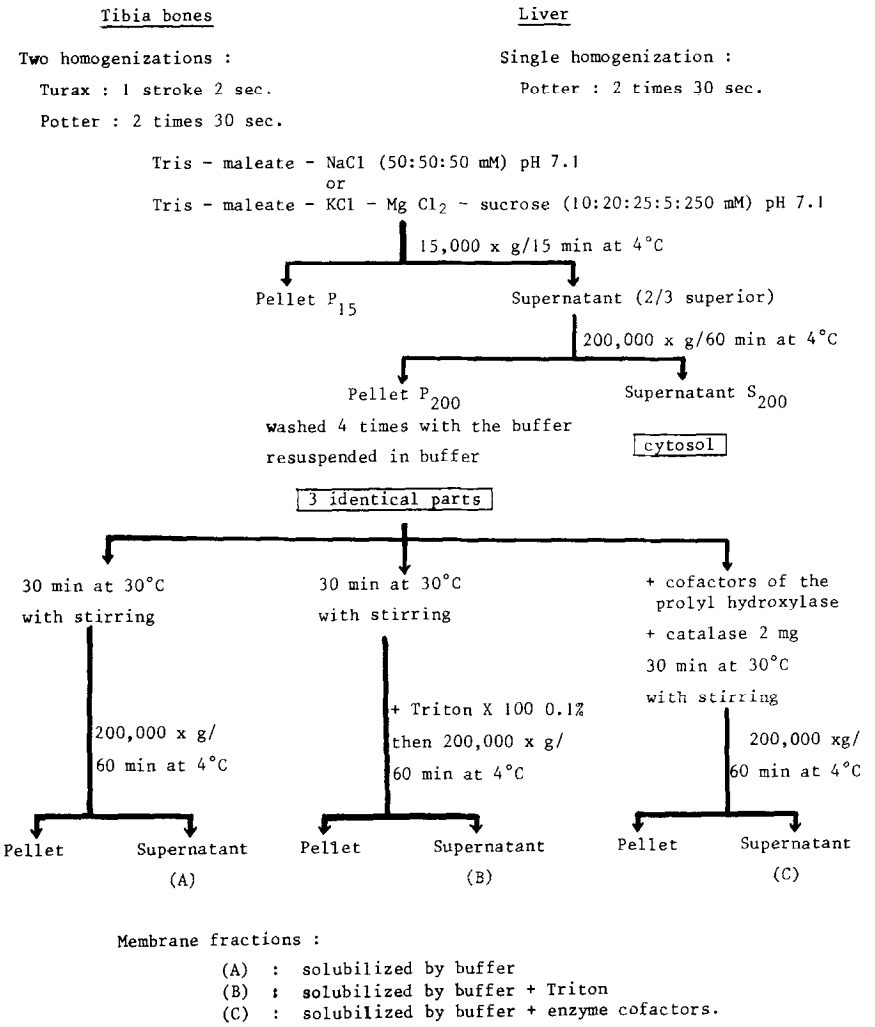
TABLE I

Fractionation in :

Tris - maleate - NaCl buffer (I)

Tris - maleate - KCl - $MgCl_2$ - sucrose buffer *(II)*

			Cytosol	Solubilized membrane fractions		
				Buffer (A)	Buffer + Triton (B)	Buffer + PPH cofactors (C)
P.P.H. Total activity (dpm ^3H_2O/20 min)	Liver	(I)	162,000	5,400	54,000	20,500
		(II)	*152,700*	*29,700*	*118,800*	*43,700*
	Tibia	(I)	157,500	9,000	18,000	18,000
		(II)	*147,800*	*31,700*	*210,500*	*96,000*
Total proteins (mg)	Liver	(I)	2,055	185	210	170
		(II)	*3,900*	*137*	*203*	*125*
	Tibia	(I)	40	30	35	30
		(II)	*79*	*5*	*13*	*6*
P.P.H. Specific activity (dpm/mg)	Liver	(I)	79	29	257	119
		(II)	*39*	*217*	*585*	*350*
	Tibia	(I)	3,937	300	514	600
		(II)	*1,871*	*6,340*	*16,192*	*16,000*



Membrane fractions :

(A) : solubilized by buffer
(B) : solubilized by buffer + Triton
(C) : solubilized by buffer + enzyme cofactors.

Figure 1

Method of subcellular fractionation
and prolyl hydroxylase solubilization

be demonstrated by solubilization using buffer only. This solubilization does not essentially proceed from membrane damage since it was obtained also when KCl - $Mg\ Cl_2$ - sucrose, which protects membrane structure, were added into the buffer (table I).

- the link between the enzyme and its membrane-bound substrate appeared when we compared the solubilization by a buffer and that obtained after treatment by a buffer containing the cofactors of the enzyme activity. These cofactors act by releasing the enzyme from its membrane-bound substrate and thus solubilizing the enzyme.

TABLE II

Ratio of each fraction to the membrane fraction extracted by Triton

Tris - maleate - NaCl buffer (I)

Tris - maleate - KCl - $MgCl_2$ - sucrose buffer *(II)*

			Cytosol	Solubilized membrane fractions		
				Buffer (A)	Buffer + Triton (B)	Buffer + PPH cofactors (C)
P.P.H. Total activity	Liver	(I)	3.00	0.10	1.00	0.38
		(II)	*1.29*	*0.25*	*1.00*	*0.37*
	Tibia	(I)	8.75	0.50	1.00	1.00
		(II)	*0.70*	*0.15*	*1.00*	*0.46*
Proteins	Liver	(I)	9.79	0.88	1.00	0.81
		(II)	*19.21*	*0.67*	*1.00*	*0.62*
	Tibia	(I)	1.14	0.86	1.00	0.86
		(II)	*6.08*	*0.38*	*1.00*	*0.46*
P.P.H. Specific activity	Liver	(I)	0.31	0.11	1.00	0.46
		(II)	***0.07***	*0.37*	***1.00***	***0.60***
	Tibia	(I)	7.66	0.58	1.00	1.17
		(II)	*0.12*	*0.39*	*1.00*	*0.99*

The enzyme activity released by cofactors corrected for activity released by buffer is an important part of the activity which occurs in the membrane fraction. The ratio of this specific activity to those obtained after triton extraction, demonstrates that approximately 30 % of the membrane-bound enzyme in the liver and 60 % of the membrane-bound enzyme in the tibia bones are bound through a membrane-bound substrate. It seems that this peptide substrate could be linked on membrane-bound polysomes or adsorbed on the membranes after its synthesis. It would be considered that using buffer without Mg^{++} and K^{+} which splits polysomes, a positive solubilization with the enzyme cofactors was still observed. This mode of binding may occur in the cisternae of the endoplasmic reticulum as shown by Olsen et al. (7) from cytochemical observations.

- the part of the enzyme activity which really occurs as a membrane component must be estimated by subtraction of the fraction solubilized by enzyme cofactors from that solubilized by Triton. It appears that, in liver, less than half of the prolyl hydroxylase is a part of the membrane structure ; but, probably, this kind of fixation does not exist in the tibia bones because the specific activity released by the enzyme cofactors is equal and sometimes greater than that released by the Triton itself.

CONCLUSION :

Subsequent to these experiments the question is whether or not prolyl hydroxylase is a membrane enzyme.

Our results show that an important part or the totality, in the case of tibia bones, of this enzyme is not directly a component of the membrane structure.

In any case, the intracellular procollagen, by binding with the enzyme, holds a part of soluble prolyl hydroxylase to the membranes which are either the membrane-bound polysomes where the procollagen is synthesized or the cisternae where it is stored before its secretion : so that a part of membrane-bound proline hydroxylase should be released soluble after enzymic reaction and before new binding with membrane-bound procollagen.

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