Labelling of prolyl hydroxylase tetrameric subunits in freshly isolated chick-embryo tendon cells and in certain chick-embryo tissues *in vivo*

Kari MAJAMAA and Jouko OIKARINEN
Department of Medical Biochemistry, University of Oulu, Kajaanintie 52A, 90220 Oulu 22, Finland

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The labelling of the subunits of prolyl 4-hydroxylase tetramers was studied in freshly isolated chick-embryo tendon cells and in chick-embryo tissues. In the former both the α- and β-subunits of the tetramer were labelled during a 4 h labelling and 2 h chase period, although the radioactivity in the β-subunit was much lower than in the α-subunit. The corresponding subunits of the enzyme from 12-day chick-embryo cartilaginous bone and heart were labelled in 7 h, again the β-subunit much less than the α-subunit, the ratio of radioactivity in the β-subunit to that in the α-subunit (β/α-radioactivity) being 0.20 and 0.32 respectively. The β/α-radioactivity then increased almost linearly with time between 7 and 24 h, by 9.5-fold in the cartilaginous bone and 3-fold in the heart, and β/α-radioactivity values above 1.0 were reached. The free β-subunit-size protein (the β'-protein), which is also present in cells, had been labelled quite heavily by 7 h. The β/α-radioactivity at 7 h, determined in four tissues with different ratios of prolyl hydroxylase tetramers to total immunoreactive protein (tetramer percentage), was low in tissues with a high tetramer percentage. It is thus proposed that only a minor fraction of the β'-protein must be processed to the tetrameric β-subunit and utilized in the synthesis of the prolyl 4-hydroxylation systems.

Prolyl 4-hydroxylase [prolyl-glycyl-peptide,2-oxoglutarate:O₂ oxidoreductase (4-hydroxylating), EC 1.14.11.2; termed here prolyl hydroxylase] is an intracellular microsomal enzyme that catalyses the formation of peptide-bound 4-hydroxyprolyl residues in the biosynthesis of collagen (for reviews, see Cardinale & Udenfriend, 1974; Prockop et al., 1976; Kivirikko & Myllylä, 1980). The enzyme is found in tissues in two forms. The active enzyme is a tetramer (M₄, 240000) with the structure α₂β₂, where α and β are dissimilar subunits with molecular weights of 64000 and 60000. There is also an inactive protein in tissues that reacts with antibodies against the tetramer. The inactive protein is related in structure to the tetrameric β-subunit, and its mobility in gel electrophoresis is identical with that of the β-subunit (Tuderman et al., 1977; Chen-Kiang et al., 1977; Kao & Chou, 1980; Berg et al., 1980). The inactive protein has been termed cross-reacting protein (McGee & Udenfriend, 1972), monomer-size protein (Tuderman et al., 1977) and β-subunit-size protein (Myllylä et al., 1981). The term β'-protein will be preferred to these in the present paper.

The ratio of active prolyl hydroxylase tetramers to total immunoreactive enzyme protein (tetramer percentage) varies in different tissues (Tuderman, 1976; Tuderman & Kivirikko, 1977) and in the same tissue during development or aging and under a number of physiological and pathological conditions (see Kivirikko & Myllylä, 1980). The metabolic relationship between the prolyl hydroxylase tetramer and the β'-protein is nevertheless not clear. Labelling of chick-embryo tissues *in vivo* showed that the β'-protein is most likely to be a precursor for the tetramer and that not all of the β'-protein is converted into the tetramer (Majamaa et al., 1979; Chichester et al., 1979). In cultured L-929 fibroblasts (Kao & Chou, 1980) and freshly isolated chick-embryo tendon cells (Berg et al., 1980) only the tetrameric α-subunit and the β'-protein are labelled during a 4 h labelling period. The tetrameric β-subunit is labelled in L-929 cells only after a chase of 24 h (Kao & Chou, 1980), indicating some kind of delay in the synthesis of the β-subunit.

In the present study the labelling of tetrameric α- and β-subunits and the β'-protein was studied *in vivo* in chick-embryo tissues with different tetramer percentages and in freshly isolated chick-embryo tendon cells in order to study further the metabolic relationship between the β'-protein and the tetrameric β-subunit.
Experimental

Materials

Fertilized eggs of White Leghorn chickens, obtained from Siipikarjanhoitajien litto r.y. (Hämeenlinna, Finland), were incubated in a moist atmosphere at 37°C until used.

L-[3,4,5-3H]Leucine (137Ci/mmol) was from Amersham International (Amersham, Bucks., U.K.). DEAE-Sephadex A-50 for ion-exchange chromatography and Sepharose 4B for the affinity columns were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). Bio-Gel A-1.5m was from Bio-Rad (Bromley, Kent, U.K.). Poly(L-proline) with Mr 30000 and 8000 was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Cells from chick-embryo leg tendons were isolated by digestion with trypsin from Grand Island Biological Co. (Grand Island, NY, U.S.A.) and bacterial collagenase from Sigma Chemical Co. (Kingston-upon-Thames, U.K.).

Preparation of the tissue and cell samples

On day 12 the developing embryos were labelled with 200μCi of [3H]leucine as described previously (Majamaa et al., 1979). Incubation was continued, and the embryos were killed 7, 14 and 24h after labelling. The humeri, ulnae, radii, femurs and tibiae (constituting the cartilaginous bone sample), the heart, the lungs and the kidneys were carefully dissected and weighed. The samples were stored at -70°C and thawed and homogenized one at a time. The cartilaginous bones were homogenized with an Ultra-Turrax homogenizer for 3 x 5s in a cold solution (0°C) consisting of 0.1M-NaCl, 0.1M-glycine, 0.1% (v/v) Triton X-100 and 10mM-Tris/HCl buffer, adjusted to pH 7.8 at 4°C (4 ml of solution/g of tissue). The heart, lung and kidney samples were homogenized in the same solution with a Teflon/glass homogenizer (50 strokes). After an incubation of 1h at 4°C with occasional stirring, the sample was centrifuged at 15000g for 30 min at 4°C. The supernatant was taken for the isolation of the tetratetramers and the β-protein.

Cells from 16-day chick-embryo leg tendons were isolated by digestion with trypsin and bacterial collagenase (Dehm & Prockop, 1971, 1972). The freshly isolated cells from about 30 embryos were then incubated in 10ml of a modified Krebs medium (Dehm & Prockop, 1971) with 200μCi of [3H]leucine for 4h. The label was chased by changing the medium, and incubation was continued for an additional 2h. The prolyl hydroxylase tetratetramers and the β-protein were then isolated.

Isolation of prolyl hydroxylase tetratetramers

The tissue or cell supernatant was applied to an affinity column of poly(L-proline) (Tuderman et al., 1975a) linked to agarose, with a bed volume of 1.0ml. The column was washed with 0.1M-NaCl/0.1M-glycine/10mM-Tris/HCl buffer (pH 7.8 at +4°C), and the bound material was eluted with 0.5ml of the above solution containing 1.5mg of poly(L-proline) of Mr 8000 (Tuderman et al., 1975a). The fractions (0.5ml) were assayed for total radioactivity. The eluted fractions with the highest radioactivities, representing the prolyl hydroxylase tetratetramers, were pooled and dialysed against water.

Isolation of the β'-protein

Rabbit immunoglobulins were precipitated with 40%-saturated (NH₄)₂SO₄ from antiserum against pure chick prolyl hydroxylase tetratetramers (Livingston, 1974). The dissolved precipitate was applied to a DEAE-Sephadex A-50 ion-exchange column equilibrated with phosphate buffer (10mM-NaH₂PO₄/10mM-Na₂HPO₄ pH 6.8 at +4°C). The effluent was then passed through a column of chick prolyl hydroxylase tetratetramers coupled to Sepharose 4B, and the column was eluted with 3M-NaSCN/10mM-phosphate buffer. The antibodies eluted were dialysed against 0.1M-NaCl/50mM-Tris/HCl (pH 7.5) and then coupled to Sepharose 4B by using the CNBr-activation technique (Cuatrecasas & Anfinsen, 1971).

Columns of volume 1.0ml were prepared from this material. The effluent from the poly(L-proline) affinity column was applied to the column and 1.0ml fractions were collected. The column was eluted with 3M-NaSCN/10mM-phosphate buffer and the fractions with the highest total radioactivity were pooled and dialysed against water. This preparation contained the β'-protein.

Both dialysed samples were freeze-dried and reconstituted in 100μl of sample buffer, containing 2% (w/v) sodium dodecyl sulphate, 0.5mM-urea, 10% (v/v) glycerol, 0.024mg of Bromophenol Blue/ml and 0.0625m-Tris/HCl, adjusted to pH 6.8 at 22°C. The samples were heated to 60°C, and after cooling 5μl of 5mM-dithiothreitol was added and the samples were subjected to sodium dodecyl sulphate/polyacrylamide-slab gel electrophoresis (King & Laemmli, 1971), in a 8% (w/v) polyacrylamide separating gel. The radioactive bands in the gel were then made visible by fluorography (Bonner & Laskey, 1974; Laskey & Mills, 1975) and scanned with a Chromafile K-495000 Densitometer (Kontes, Vineland, NJ, U.S.A.).

Other assays

The relative amounts of prolyl hydroxylase tetratetramers and the β'-protein were studied in non-labelled 12-day chick embryos. The tissue samples were homogenized as described above and the supernatant was applied to a gel-filtration column (1.5cm x 90cm; Bio-Gel A-1.5m). The fractions
were assayed for the immunoreactive prolyl hydroxylase protein (Tuderman et al., 1975b), and the ratio of immunoreactive tetramers to total immunoreactive protein (tetramer percentage) was calculated.

The samples were dissolved in 5 ml of Lumagel (Lumac Systems A.G., Basel, Switzerland) for counting for \(^3\)H radioactivity in a Wallac liquid-scintillation spectrometer with an efficiency of 35% and background of 10 c.p.m.

**Results**

\(\beta/\alpha\)-Radioactivity in freshly isolated chick-embryo tendon cells

Tendon cells from 16-day chick embryos were isolated and the freshly isolated cells from 30 embryos incubated with 200 \(\mu\)Ci of \([^{3}\text{H}]\)leucine for 4 h in 10 ml of modified Krebs solution. The label was then chased for an additional 2 h. The cells were homogenized and the prolyl hydroxylase tetramers and \(\beta\)-protein were isolated and subjected to slab gel electrophoresis. The fluorograph of the gel was scanned (Fig. 1). No kinetic studies were performed on these cells, but the validity of the method was tested. Fig. 1 shows the constant finding in fluorographs that the tetrameric \(\beta\)-subunit and the \(\beta\)\(^{\prime}\)-protein have an identical mobility, which is different from that of the standard tetrameric \(\beta\)-subunit labelled with \(^3\)H by using a technique of reductive alkylation with formaldehyde and \(\text{NaB}_3\text{H}_4\) (Tuderman et al., 1975b). The non-labelled \(\beta\)-subunit and the \(\beta\)-subunit labelled \textit{in vivo} had identical mobilities, however (results not shown). Both subunits of the tetramer were labelled in the experiment with freshly isolated cells (Fig. 1), the radioactivity in the \(\beta\)-subunit being low, but clearly detectable. The \(\alpha\)-subunit and \(\beta\)-protein were labelled quite heavily, and the \(\beta/\alpha\)-radioactivity (ratio of radioactivity in \(\beta\)-subunit to that in \(\alpha\)-subunit) in this experiment was 0.16.

![Fluorograph](image)

**Fig. 1. Labelling of tetrameric \(\alpha\)- and \(\beta\)-subunits and the \(\beta\)\(^{\prime}\)-protein in freshly isolated chick-embryo tendon cells**

Freshly isolated tendon cells were labelled for 4 h with 200 \(\mu\)Ci of \([^{3}\text{H}]\)leucine and the label was chased for 2 h. Prolyl hydroxylase tetramers and the \(\beta\)-protein were isolated and electrophoresed on a sodium dodecyl sulphate/polyacrylamide slab gel. (a) Fluorograph exposed on the gel. Lanes 1 and 3, \(^3\)H-labelled prolyl hydroxylase tetramer standard dissociated with dithiothreitol before application to the gel; lane 2, cellular \(\beta\)-protein; lane 4, cellular prolyl hydroxylase tetramer dissociated with dithiothreitol. (b) Scan from lane 4 of the above fluorograph. \(\alpha\) and \(\beta\) denote the migration of standard tetrameric \(\alpha\)- and \(\beta\)-subunits respectively; F denotes the front.
Fig. 2. β/α-Radioactivity in prolyl hydroxylase tetramers in chick-embryo cartilaginous bone and heart

[1H]Leucine (200 µCi) was injected on to the chorioallantoic membrane on day 12 of chick-embryo development. The embryos were killed at the time points indicated and the cartilaginous bones and heart were dissected out. Prolyl hydroxylase tetramers and the β-protein were isolated and the resulting material was used for sodium dodecyl sulphate/polyacrylamide-slab-gel electrophoresis. The fluorograph from the gel was then scanned and the peak areas for tetrameric α- and β-subunits were calculated. The ratio of the β-subunit area to the α-subunit area (β/α-radioactivity) is shown for cartilaginous bone (a) and heart (b).

β/α-Radioactivity in certain chick-embryo tissues

The tetramer percentage was assayed preliminarily in 12-day chick embryos. The percentages (Fig. 3) were in accordance with those reported previously (Tuderman, 1976). The tissues with [3H]leucine label were dissected 7, 14 and 24 h after the introduction of radioactivity to the chorioallantoic membrane of the developing embryo. The dissected tissues, the kidney, lung, heart and cartilaginous bone, were selected to give a range of tetramer percentage of 18–56%.

Prolyl hydroxylase tetramers and β-protein were isolated from the cartilaginous bone and heart tissues and assayed at each of the above time points. The α- and β-subunits of the tetramer were labelled during the first 7 h in both tissues, but the radioactivity was lower in the β-subunit than in the α-subunit, β/α-radioactivity being 0.20 in cartilaginous bone and 0.32 in heart (Fig. 2). The β-protein was also labelled at this point (results not shown). The β/α-radioactivity increased almost linearly with time, and reaching 1.90 at 24 h in the cartilaginous bone and 1.02 in the heart (Fig. 2).

The radioactivity of the α- and β-subunits was assayed at 7 h in four tissues with different tetramer percentages (Fig. 3). β/α-Radioactivity was lower in the tissues with a higher tetramer percentage at this point, although later it was higher in tissues with a higher tetramer percentage. This latter finding was based only on the β/α-radioactivity determinations from cartilaginous bone and heart, however (Fig. 2).

Discussion

Prolyl hydroxylase is found in tissues in two forms: the active enzyme tetramer and an inactive β-protein (see Kivirikko & Myllylä, 1980). Previous studies on the metabolic relationship between the β-protein and the tetrameric β-subunit have suggested a precursor–product relationship (Majamaa

1982
et al., 1979; Chichester et al., 1979; Kao & Chou, 1980; Berg et al., 1980). The present results support this relationship, and moreover suggest that only a minor fraction of the total \( \beta' \)-protein is utilized for processing to tetrameric \( \beta \)-subunits.

The present data show that the labelled amino acid enters tetrameric \( \beta \)-subunit later than \( \alpha \)-subunit in vivo. The method used required incorporated radioactivities that were achieved only after 7h labelling, at which point the \( \beta' \)/\( \alpha' \)-radioactivity was low, but the radioactivity in the \( \beta \)-subunit was nevertheless clearly detectable. The \( \alpha \)-subunit and the \( \beta' \)-protein were labelled quite heavily at this point. In freshly isolated chick-embryo tendon cells, radioactivity was found only in the \( \alpha \)-subunit after a 4h pulse, even after an additional chase period of 4h (Berg et al., 1980). Here a similar experiment, but with a chase period of 2h, showed labelling of the \( \beta \)-subunit with a low \( \beta' \)/\( \alpha' \)-radioactivity, 0.16. It seems therefore that there is a lag period of 4–6h in the synthesis of the \( \beta \)-subunit or its assembly into the tetramer, a delay unlikely to be due to differences in assembly. A more probable explanation is that \( \beta \)-subunit is processed from a precursor.

The \( \beta' \)-protein is labelled rapidly in vivo in chick-embryo cartilaginous bone (Majamaa et al., 1979) and in vitro in freshly isolated chick-embryo tendon cells (Berg et al., 1980), and the specific-radioactivity decay curves for chick-embryo cartilaginous bone suggest that the \( \beta' \)-protein behaves more like a precursor than a degradation product (Majamaa et al., 1979). The same conclusion has also been reached on the basis of labelling experiments with cultured (Kao & Chou, 1980) and freshly isolated (Berg et al., 1980) fibroblasts. The structures of the \( \beta \)-subunit and the \( \beta' \)-protein show great similarity, but they nevertheless differ in two out of 37 tryptic peptides (Chen-Kiang et al., 1977) and in at least one out of about 15 V8-protease peptides (Berg et al., 1980). The processing of the \( \beta' \)-protein to the \( \beta \)-subunit may thus be the rate-limiting step in the introduction of label into the tetrameric \( \beta \)-subunit, although the nature of such a mechanism is unknown.

The \( \beta' \)/\( \alpha' \)-radioactivity increased almost linearly during the first 24h and still showed an upward trend at this point. The increase between 7 and 24h was 9.5-fold in cartilaginous bone and 3-fold in heart tissue. The half-life of prolyl hydroxylase tetramers in chick-embryo cartilaginous bone is about 39h when measured after labelling of the embryos in vivo (Majamaa et al., 1979). The high \( \beta'/\alpha' \)-radioactivities cannot be explained merely by the turnover of the rapidly labelled \( \alpha \)-subunit. The labelling of the \( \alpha \)-subunit in tissues becomes slower as the free amino acid pool decreases (Poole et al., 1971; Waterlow et al., 1978; Majamaa et al., 1979). The increase in \( \beta'/\alpha' \)-radioactivity suggests that \( \beta \)-subunit is synthe-

sized from a pool, the specific radioactivity of which exceeds that of the tetrameric \( \alpha \)-subunit. The \( \beta'/\alpha' \)-radioactivities at 7h were higher in those tissues with lower tetramer percentages. This finding would be consistent with the hypothesis that the \( \beta' \)-protein destined for processing and the processed \( \beta \)-subunit form a relatively small pool that is part of the total \( \beta' \)-protein (see below). This small pool would make possible the high specific radioactivity that is apparently required for the rapid increase found in \( \beta'/\alpha' \)-radioactivity (Fig. 2). This hypothesis is supported by calculations in a dynamic model shown in Fig. 4. Since the synthesis of an enzyme follows zero-order kinetics (Schimke & Doyle, 1970; Rechcigl, 1971), the size of this pool is related to the rate of tetramer synthesis and the size of the tetramer pool. Interestingly, the integration of the \( \beta' \)-protein into cellular plasma membranes has been demonstrated (Kao & Chou, 1980). According to previous results, prolyl hydroxylase tetramers are located within the cisternae of the rough endoplasmic reticulum or loosely bound to its inner membrane, whereas another enzyme of collagen biosynthesis, lysyl hydroxylase, is more distinctly bound to the membranes (Kivirikko & Myllylä, 1980). It is tempting to postulate that the unprocessed fraction of the \( \beta' \)-protein may be transferred to another cellular compartment, so that it is no longer available for processing or assembly into tetramers. This would also explain the small \( \beta' \)-pool (Fig. 4). The unprocessed fraction comprises the majority of the total \( \beta' \)-protein, but its function, if any, is unknown.

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**Fig. 4. Dynamic model for the relationship between the \( \beta' \)-protein and the prolyl hydroxylase tetramer**

The hypothesis put forward in the Discussion section is based on the data presented and on model calculations. For the calculations it was assumed that both proteins have a half-life of 39h (Majamaa et al., 1979) and that the system is in a steady state. The \( \beta'/\alpha' \)-radioactivity in the tetramers was calculated and the results agreed with experimental data in the above model. The pool sizes are arbitrary. \( K_r \), rate constant of synthesis; \( K_d \), rate constant of degradation; \( \beta_n \), newly synthesized \( \beta' \)-protein available for processing; \( \beta' \), the \( \beta' \)-protein; \( \beta \), the \( \beta \)-subunit; the wavy line between \( \beta_n \) and \( \beta \) indicates processing; \( T \), tetramer with structure \( \alpha_{3} \beta_{2} \).
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