The assembly of tetrameric prolyl hydroxylase in tendon fibroblasts from newly synthesized α -subunits and from preformed cross-reacting protein

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Embryonic-chick tendon cells were incubated in suspension for 4 h with ^{14}C -labelled amino acids, cell extracts were subjected to gel filtration, and the effluent was examined by rocket immunoelectrophoresis by using antibodies specific for the β -subunit of chick prolyl hydroxylase. Two peaks of immunoreactive protein were found. The first peak contained 40% of the immunoreactive protein eluted from the column and 100% of the enzyme activity. Polyacrylamide-slab-gel electrophoresis in sodium dodecyl sulphate of an immunoprecipitate of this peak demonstrated that it consisted of the tetrameric form of prolyl hydroxylase, subunit composition $\alpha_2\beta_2$ where α and β are non-identical subunits. Only the α -subunits were labelled, indicating that they were synthesized during the 4 h labelling period. The β -subunits were unlabelled, indicating that they had been synthesized before the labelling period. The second peak eluted from the gel-filtration column contained 60% of the immunoreactive protein eluted from the column and was enzymically inactive. Polyacrylamide-slab-gel electrophoresis of an immunoprecipitate of this peak indicated that it consisted of a single labelled polypeptide chain, identified as cross-reacting protein, which was related to, but not identical with, the β -subunit of prolyl hydroxylase. Pulse-chase experiments were performed on cultured chick tendon cells to demonstrate that α -subunits and cross-reacting protein had half-lives of about 60 h. The half-life of β -subunits was considerably longer, and the kinetic pattern was consistent with their being derived from a labelled precursor such as cross-reacting protein. The data presented here indicate that the active tetrameric form of prolyl hydroxylase in cells is assembled from α -subunits which are newly synthesized, and from β -subunits which are derived from cross-reacting protein.

Prolyl hydroxylase [prolyl-glycyl-peptide,2-oxo-glutarate oxidoreductase (4-hydroxylating), EC 1.14.11.2] has been purified to homogeneity from newborn rats (Rhoads & Udenfriend, 1970; Risteli *et al.*, 1976), chick embryos (Berg & Prockop, 1973), human foetal skin (Kuutti *et al.*, 1975) and cultured L-929 fibroblasts (Kao & Chou, 1978; Kao & Berg, 1979). It is a microsomal enzyme which is located in the endoplasmic reticulum of fibroblasts, where it is involved in collagen biosynthesis (Prockop *et al.*, 1976). The active form of the enzyme has been shown to be a tetramer composed of two pairs of non-identical subunits, α and β , in a 1:1 ratio (Berg & Prockop, 1973; Berg *et al.*, 1979). An enzymically inactive form of the enzyme has been

identified immunologically in many different cells and tissues (McGee *et al.*, 1971; McGee & Udenfriend, 1972; Stassen *et al.*, 1974; Kao *et al.*, 1975; Tuderman & Kivirikko, 1977; Chen-Kiang *et al.*, 1977; Risteli *et al.*, 1978), and has been referred to as cross-reacting protein (McGee *et al.*, 1971). Cross-reacting protein was purified from rat skin and cultured L-929 fibroblasts and was shown to be a single polypeptide chain related to only the β -subunit (Chen-Kiang *et al.*, 1977; Kao & Berg, 1979). Little information is known about the relationship of cross-reacting protein to the active form of prolyl hydroxylase. It has been observed that the specific radioactivity of cross-reacting protein was higher than that of tetramers in lung and cartilaginous bone of chick embryos, suggesting that it was metabolically active and not a degradation product derived from active enzyme (Majamaa *et al.*, 1979). In the present work antibodies specific for

Abbreviation used: SDS, sodium dodecyl sulphate.

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the β -subunit were used to examine the kinetics of incorporation of labelled amino acids into α - and β -subunits and cross-reacting protein and to explore the function of cross-reacting protein in the assembly of the active form of prolyl hydroxylase.

A preliminary report of this study was presented at the 18th Annual meeting of the American Society for Cell Biology, November 4–8, 1978, San Antonio, Texas (Berg & Kao, 1978).

Experimental

Freshly isolated tendon cells

To prepare tendon cells, leg tendons were removed from 17-day chick embryos and cells were obtained from the tendons by controlled digestion with trypsin and collagenase (Dehm & Prockop, 1972; Kao *et al.*, 1977).

For analysis of intracellular forms of prolyl hydroxylase, the cells were incubated in suspension at a concentration of 1×10^9 cells in 40 ml of modified Krebs medium II (Dehm & Prockop, 1971) containing 400 μ Ci of 14 C-labelled amino acid mixture (sp. radioactivity 0.6 mCi/mg; New England Nuclear, Boston, MA, U.S.A.) for 4 h at 37°C. To chase the radioactive label, the cell suspension was centrifuged for 5 min at 700 *g* to separate cells from medium and the cells were resuspended in 40 ml of modified Krebs medium II containing 10% foetal calf serum. The incubation was continued for 4 h, at which time the cells were harvested by centrifugation as above. Cell extracts were obtained by resuspending the cell pellets in 4.0 ml of 0.01 M-Tris/HCl buffer, pH 7.8 at 4°C, containing 0.2 M-NaCl, 0.1 M-glycine, 10 μ M-dithiothreitol and 0.1% (v/v) Nonidet P40 (Shell Chemicals, Bethesda Research, Bethesda, MD, U.S.A.). The cells were homogenized with ten strokes in a Teflon/glass homogenizer and centrifuged at 20 000 *g* for 30 min to remove the cell debris. These conditions were previously shown to extract maximal amounts of total immunoreactive prolyl hydroxylase and prolyl hydroxylase activity from freshly isolated tendon cells (Kao *et al.*, 1975). Portions of cell extracts were chromatographed on a column (0.9 cm $\times$ 45 cm) of Ultrogel AcA-34 (LKB) or Agarose A-1.5 M (Bio-Rad Laboratories). The column fractions were assayed for prolyl hydroxylase activity with α -oxo [1- 14 C]glutarate (New England Nuclear) as one substrate and (Pro-Pro-Gly)₁₀ (100 μ g/ml; Protein Research Foundation, Osaka, Japan) as the second substrate. Values of enzyme activity were converted into units, which were defined as the amount of enzyme required to synthesize 1 μ g of hydroxyproline/h at 37°C (Berg & Prockop, 1973). Column fractions were also assayed for immunoreactive protein by using rocket immunoelectrophoresis (see below).

Primary cultures of freshly isolated tendon cells

To prepare primary cultures, tendon cells were obtained as described above, but under sterile conditions. The cells were grown in Dulbecco's medium supplemented with 10% foetal calf serum, 100 units of penicillin/ml and 100 μ g of streptomycin/ml in an atmosphere of CO₂/air (1:9) at 37°C. Cells at mid-exponential phase in the second passage were harvested by trypsin treatment and stored in liquid N₂ as described previously (Kao *et al.*, 1976). To label cells with [3 H]leucine, cells stored in liquid N₂ were thawed quickly in a water bath at 40°C by vigorous shaking and then were inoculated in Falcon plastic culture flasks (150 cm²) at 5×10^6 cells/flask. The cells were grown to mid-exponential phase and then rinsed three times with serum-free Dulbecco's medium without leucine. The cultures were then labelled with [3 H]leucine at 10 μ Ci/ml in the same medium at 37°C for 4 h. The [3 H]leucine label was chased by replacing the radioactive medium with fresh medium containing 105 μ g of unlabelled [3 H]leucine/ml and 10% (v/v) foetal calf serum for 6–48 h. At each point the cells were harvested by trypsin treatment and lysed in lysis buffer, containing 0.5% (v/v) Nonidet P40, 50 μ g of deoxyribonuclease/ml, 50 μ g of ribonuclease/ml, 1 mM-MgCl₂ and 0.01 M-Tris/HCl, pH 7.5 at 4°C, for 30 min. The lysates were centrifuged at 20 000 *g* for 20 min to remove cell debris, and the supernatants were chromatographed on a column (0.9 cm $\times$ 30 cm) of Ultrogel AcA-34. The tetramer and monomer peaks of prolyl hydroxylase were identified by rocket immunoelectrophoresis and subjected to immunoprecipitation (see below). The immunoprecipitates were treated with 0.5 ml of sample buffer, containing 2% (w/v) SDS, 10% (v/v) glycerol, 0.001% Bromophenol Blue and 0.125 M-Tris/HCl, pH 6.8, and subjected to polyacrylamide-gel electrophoresis in cylindrical gels of 10% (w/v) polyacrylamide as described below. The gels were stained with Coomassie Brilliant Blue R, destained and scanned at 560 nm in a Gilford spectrophotometer equipped with a linear transport. The gels were then sliced on a gel slicer (Mickle), and each slice was dissolved in 0.5 ml of Protosol (New England Nuclear) and counted for radioactivity in a scintillation counter. The total amount of protein in each stained band of subunit of active enzyme and of cross-reacting protein was determined by comparing the staining intensity with that of standards, and the amount of 14 C-labelled protein was obtained by counting the radioactivity of the sliced gels. The specific radioactivity of each subunit in the tetramer and of cross-reacting protein was calculated. To determine the total radioactivity in each polypeptide chain, the specific radioactivity of each polypeptide chain was multiplied by the total amount of each polypeptide chain obtained from the peaks of

immunoreactive polypeptide eluted from the AcA-34 column. The radioactivity in each polypeptide was calculated as the percentage of the radioactivity present in the polypeptide at the end of the labelling period. A first-order semi-log plot of the percentage of radioactivity remaining in each peptide during the chase period was constructed, and the half-lives for the disappearance of labelled polypeptide were obtained from the graph (Schimke & Doyle, 1970). The radioactivity in total cellular protein was determined by precipitating a sample of the cell extract with 10% (w/v) trichloroacetic acid at various times during the chase. The precipitates were washed twice with 10% trichloroacetic acid to remove non-protein-bound [^3H]leucine and dissolved in 0.5 ml of NCS tissue solubilizer (Amersham Corp., Arlington Heights, IL, U.S.A.) before radioactivity counting. The radioactivity in the precipitates obtained at various time points was calculated as a percentage of that in the precipitate at the end of the labelling period.

Immunological procedures

Prolyl hydroxylase was purified from chick embryos (Berg & Prockop, 1973) and antibodies were prepared as described previously (Berg *et al.*, 1972; Harboe & Ingild, 1973). For comparison of antigenicity of α - and β -subunits, the subunits were prepared from purified prolyl hydroxylase (Berg *et al.*, 1979) and were made to react separately with antibodies by immunodiffusion and rocket immunoelectrophoresis (Weeke, 1973).

Immunoprecipitation of cell extracts and fractions of cell extract after gel filtration was performed by adding 0.4 ml of sample containing approx. 40 μg of immunoreactive protein and 0.1 ml of an immunoglobulin G fraction of specific antisera against chick prolyl hydroxylase. The volume was adjusted to 1 ml with solution containing 0.5% (v/v) Nonidet P40, 0.5% (v/v) sodium deoxycholate (Sigma Chemical Co.), 0.135 M-NaCl and 0.01 M-sodium phosphate buffer, pH 7.5. The samples were incubated at 37°C for 1 h and then at 4°C overnight. The immunoprecipitates were layered over 0.5 ml of the phosphate buffer described above, containing 1 M-sucrose, 10 mM-EDTA, 0.5% (v/v) sodium deoxycholate, 0.5% (v/v) Nonidet P40 (Palmiter, 1973) and centrifuged for 15 min in a table-top centrifuge (model 3200, Brinkmann) at 8000 g. The pellets were resuspended in 0.1 ml of 5 M-urea containing 5% (w/v) SDS and dialysed against sample buffer (see above).

Polyacrylamide-gel electrophoresis of immunoprecipitates was performed by the method of King & Laemmli (1971) as described previously (Kao *et al.*, 1977) in 0.1 cm $\times$ 10 cm $\times$ 15 cm slabs of polyacrylamide or in 0.5 cm $\times$ 10 cm cylindrical gels prepared with the same buffers used for slabs (see above).

Slab gels were dried and subjected to fluorography as previously described (Kao *et al.*, 1977). Crossed rocket immunoelectrophoresis was performed by the method of Chua & Blomberg (1979). Peptide 'maps' of bands from SDS/polyacrylamide slab gels were obtained by the method of Cleveland *et al.* (1977), as described previously (Berg *et al.*, 1979). The gels were stained with Coomassie Brilliant Blue R and then dried on to either filter paper for fluorography or clear cellulose sheets (Bio-Rad Laboratories) for photography. The gels were photographed through a green filter (X1, Nikon).

Results

Specificity of antisera for prolyl hydroxylase

Purified prolyl hydroxylase was injected into rabbits to prepare antisera. The antisera had been previously tested by immunodiffusion and immunoelectrophoresis and found to react with the native tetrameric form of prolyl hydroxylase (Berg *et al.*, 1972). More recently, subunits of prolyl hydroxylase have been prepared (Berg *et al.*, 1979), and so it

Fig. 1. Immunodiffusion of the α -subunit, the β -subunit and cross-reacting protein with antisera prepared against native prolyl hydroxylase

The α - and β -subunits of prolyl hydroxylase were purified from chick embryos as described previously (Berg *et al.*, 1979) and cross-reacting protein from embryonic-chick tendon cells was separated from the tetrameric form of prolyl hydroxylase as described in the Experimental section. Each of the peptides was dissolved in 0.2 M-NaCl/0.1 M-glycine/0.1 M-Tris/HCl, pH 7.8. Wells 1 and 2 each contained 9 μg of purified β -subunit, wells 3 and 4 each contained about 6 μg of cross-reacting protein and wells 5 and 6 each contained 10 μg of purified α -subunit. The centre well contained 30 μl of whole rabbit antiserum produced against native prolyl hydroxylase.

was possible to determine if the subunits were equally reactive with antisera. Antibodies were analysed by immunodiffusion and rocket immunoelectrophoresis and found to react with purified β -subunits, but not with purified α -subunits (Fig. 1).

Intracellular forms of prolyl hydroxylase

When extracts were prepared from freshly isolated tendon cells and subjected to gel filtration, prolyl hydroxylase activity was eluted as a symmetrical peak in the centre of the column profile (Fig. 2). When the column fractions were assayed for immunoreactive protein by using rocket immunoelectrophoresis, two peaks of immunoreactive protein were observed. The first peak contained 40% of the immunoreactive protein and corresponded to active enzyme. The second peak contained 60% of the immunoreactive protein, had a lower molecular weight, and corresponded to cross-reacting protein previously identified in fibroblasts (Stassen *et al.*, 1974; Kao *et al.*, 1975). Since the antibodies were specific for only the β -subunit derived from the active form of the enzyme, and since cross-reacting protein is related to β -subunit (see below), the results indicated that the ratio (w/w) of cross-reacting protein to β -subunit is 1.5 : 1.

Biosynthesis of prolyl hydroxylase

To label the intracellular forms of prolyl hydroxy-

lase, freshly isolated tendon cells were incubated for 4 h with a ^{14}C -labelled amino acid mixture, cell extracts were prepared and the extracts were chromatographed on a gel-filtration column as described in Fig. 2. The peaks corresponding to active enzyme and cross-reacting protein were pooled separately, subjected to immunoprecipitation and examined by polyacrylamide-slab-gel electrophoresis under denaturing conditions in SDS (Fig. 3). Staining of the immunoprecipitates demonstrated the presence of both subunits of prolyl hydroxylase in the cell extract applied to the gel-filtration column (Fig. 3, lane 2, bands B and C). The stained band D (Fig. 3, lane 2) corresponded to the heavy chain of immunoglobulin G, which is present in the immunoprecipitate. For comparison, prolyl hydroxylase was partially purified from chick embryos and subjected to polyacrylamide-slab-gel electrophoresis (Fig. 3, lane 1). The α - and β -subunits (Berg *et al.*, 1979) correspond to bands B and C in lane 2. The pooled fractions from the gel-filtration column containing active enzyme (see Fig. 2) also contained both α - and β -subunits (as shown in Fig. 3, lane 3). The α -subunit (band B) was consistently observed to appear as a doublet by staining. This subunit has been shown to be glycosylated (Berg *et al.*, 1979), and two forms of the subunit are present in cells (Kedersha *et al.*, 1980). The pooled cross-reacting protein

Fig. 2. Gel filtration of a cell extract of unlabelled chick tendon cells

Freshly isolated tendon cells were prepared as described in the Experimental section, except that the cells were not incubated before being homogenized. Then 2.5×10^8 cells were homogenized in 1.5 ml of buffer as described. The cell extract was chromatographed on a column of Ultrogel AcA-34. Fractions (1 ml) were collected and their A_{280} was measured (—). Samples (20 μl) of each fraction were used to determine prolyl hydroxylase activity (O) by using α -oxo[1- ^{14}C]glutarate as a substrate, and 10 μl of each fraction was used to determine immunoreactive protein (●) by rocket immunoelectrophoresis as described in the Experimental section.

Fig. 3. Immunoprecipitation of cell extracts of freshly isolated chick tendon cells

Freshly isolated tendon cells were prepared and labelled as described in the Experimental section. Prolyl hydroxylase was also partially purified from chick embryos by using an affinity column containing poly-L-proline (Tuderman *et al.*, 1975). The eluate from the affinity column was not subjected to gel filtration, so that another protein was present in addition to the α - and β -subunits from prolyl hydroxylase. Partially purified chick enzyme, cell extracts and cell extracts after gel filtration (Fig. 2) were immunoprecipitated and subjected to polyacrylamide-slab-gel electrophoresis in SDS. Lane 1, partially purified chick prolyl hydroxylase. Lane 2, immunoprecipitate of a cell extract of tendon cells labelled for 4 h. Lane 3, immunoprecipitate of active tetrameric form of enzyme from gel-filtration column of the cell extract of tendon cells labelled for 4 h. Lane 4, immunoprecipitate of cross-reacting protein from the gel-filtration column of the cell extract of tendon cells labelled for 4 h. Lane 5, immunoprecipitate of cell extract of tendon cells labelled for 4 h, then chased for 4 h. Lane 6, immunoprecipitate of the active tetrameric form of enzyme from the gel-filtration column of the cell extracts of tendon cells labelled for 4 h and then chased for 4 h. Lane 7, immunoprecipitate of cross-reacting protein from the gel-filtration column of cell extract labelled for 4 h and then chased for 4 h. Lane 8, immunoprecipitate of partially purified prolyl hydroxylase from lane 1. A-E indicate the stained bands of protein.

(Fig. 2, lane 1) and is a protein unrelated in structure to either of the subunits of prolyl hydroxylase (see below). When present with prolyl hydroxylase it can be partially precipitated with antibodies (Fig. 3, lane 8), but by itself it does not react with antisera to prolyl hydroxylase.

The pattern of radioactive bands in the immunoprecipitates in Fig. 3 is shown in Fig. 4. In the immunoprecipitates of the cell extract (Fig. 4, lane 2), both subunits of prolyl hydroxylase (bands B and C) were labelled, as well as the band of higher mobility (band E). The bands (Fig. 4) in the neighbourhood of band A correspond to collagen, since they are digestible with bacterial collagenase. The protein of higher mobility than prolyl hydroxylase (band E) was also labelled (see above), but was not analysed

Fig. 4. Fluorograph of the slab gel presented in Fig. 3. The slab was impregnated with 2,5-diphenyloxazole, dried and exposed to X-ray film and the film was photographed. See Fig. 3 for explanation of legend. A-C and E represent labelled bands of protein.

from the gel-filtration column (see Fig. 2) contained only the smaller or β -subunit (Fig. 3, lane 4). The fast-moving band appearing in the immunoprecipitate of the cell extract (Fig. 3, lane 2, band E) is present in partially purified prolyl hydroxylase

further. The labelling pattern in the immunoprecipitates of the active form of enzyme was quite striking in that only the α -subunit was labelled. This subunit appeared as a triplet, probably because of partial glycosylation (see above). The β -subunit in the active form of the enzyme (lane 3, band C) was unlabelled, but the cross-reacting protein (lane 4, band C) was labelled. Since the active form consists of associated subunits, $\alpha_2\beta_2$, the results indicate that the active enzyme assembled during the 4 h labelling period incorporated preformed unlabelled β -subunits. To explore the assembly process further, cells were labelled for 4 h and then chased for 4 h in the presence of ^{14}C -labelled amino acids. Immunoprecipitates of cell extract, active enzyme and a cross-reacting protein were subjected to slab-gel electrophoresis (Fig. 3 and 4). The results indicated that the subunits in the cell extract were both labelled (Fig. 4, lane 5) and the β -subunit in the active form was still unlabelled (Fig. 4, lane 6), indicating that during the 4 h chase period β -subunits that were being utilized for the assembly of tetramers were synthesized before the labelling period, or at least 8 h before the cells were prepared for analysis. Since the synthesis of labelled enzyme was so slow in freshly isolated tendon cells, cultured cells were used to study the biosynthesis of β -subunit in tetramers of prolyl hydroxylase (see below).

The relationship between cross-reacting protein and β -subunits was explored by comparing linear peptide 'maps' of each of the proteins produced by digestion with *Staphylococcus aureus* V8 proteinase. The results (Fig. 5) indicated that cross-reacting protein was very similar to but not identical with the β -subunit from the active form of prolyl hydroxylase, as had been previously shown (Chen-Kiang *et al.*, 1977; Kao & Berg, 1979). It is apparent that cross-reacting protein and β -subunit differ from one another by at least one peptide, as had previously been indicated (Chen-Kiang *et al.*, 1977).

In order to rule out the possibility that one of the bands (A-E) of protein other than cross-reacting protein in Figs. 3 and 4 could serve as a precursor of β -subunits, each of the bands (A-E) was cut out of the polyacrylamide slab gel and was subjected to peptide 'mapping' (Cleveland *et al.*, 1977). The results indicated that bands A, B and E did not share peptides with the β -subunit or cross-reacting protein (Fig. 6). Band D did not appear in Fig. 6, lane 4, because it corresponded to the heavy chain of immunoglobulin G and was not labelled. In order to demonstrate that none of the peptides in the immunoprecipitate other than β -subunits or cross-reacting protein reacted directly with antibodies to prolyl hydroxylase, an extract of tendon cells was subjected to immunoprecipitation and polyacrylamide-slab-gel electrophoresis in SDS, and then examined by crossed immunoelectrophoresis. The

Fig. 5. Linear peptide 'maps' of β -subunits and cross-reacting protein

β -Subunits and cross-reacting protein were isolated from tendon cells by gel filtration and immunoprecipitation as described in the legend to Fig. 3. Bands corresponding to β -subunits and cross-reacting protein were cut from polyacrylamide slab gels similar to those shown in Figs. 1 and 2. The polyacrylamide slices were digested with *S. aureus* V8 proteinase as described previously (Berg *et al.*, 1979) and subjected to SDS/polyacrylamide-gel electrophoresis (15% acrylamide). The gels were stained and photographed as described in the Experimental section. Lane 1, β -subunit; lane 2, cross-reacting protein. The arrow indicates the presence of a peptide in lane 2 that is not present in lane 1.

results demonstrated that band C, but not bands A, B, D and E, reacted with the antibodies directed against prolyl hydroxylase (Fig. 7). The reaction

Fig. 6. Linear peptide 'maps' of labelled proteins in an immunoprecipitate of a tendon cell extract

An immunoprecipitate of an extract of tendon cells was prepared as described in the Experimental section and subjected to polyacrylamide slab-gel electrophoresis in SDS as shown in Fig. 3, lane 2. The gel was stained briefly with Coomassie Brilliant Blue R and the bands A-E were cut out, digested with *S. aureus* V8 proteinase as described previously (Berg *et al.*, 1979) and subjected to SDS/polyacrylamide-slab-gel electrophoresis (15% acrylamide). The gel was dried and subjected to fluorography as described previously (Berg *et al.*, 1979). Lane 1, band A; lane 2, band B; lane 3, band C; lane 4, band D; lane 5, band E.

between antibodies to prolyl hydroxylase and band C is consistent with the specificity of the antisera for the β -subunit and cross-reacting protein (see Fig. 1).

Under the conditions used here for immunoprecipitation, not only are prolyl hydroxylase and

Fig. 7. Crossed immunoelectrophoresis of proteins in an immunoprecipitate of a tendon cell extract

An immunoprecipitate of an extract of tendon cells was prepared as described in the Experimental section and subjected to polyacrylamide-slab-gel electrophoresis in SDS as shown in Fig. 3. The entire lane was cut from the slab gel and electrophoresed into a flat plate of agarose containing purified rabbit antibodies directed against prolyl hydroxylase (50 μ g/ml). The agarose was dried and stained by the method of Weeke (1973). A parallel lane identical with the first was cut from the polyacrylamide slab gel, stained and photographed as described in the Experimental section. The stained polyacrylamide gel is shown with the top of the gel to the left (cf. Fig. 3, lane 2). Above the polyacrylamide gel is a photograph of the agarose gel, which was electrophoresed with the anode at the top.

cross-reacting protein precipitated (Figs. 3 and 4), but collagen (band A) and an additional protein (band E) are also precipitated. Since prolyl hydroxylase binds to collagen, which in turn binds other proteins (e.g. lysyl hydroxylase), this is not surprising. Separation by SDS/polyacrylamide-slab-gel electrophoresis followed by crossed immunoelectrophoresis demonstrates that none of these proteins react directly with the antibodies (Fig. 7).

Determination of the turnover times for α - and β -subunit and cross-reacting protein in cultured tendon cells

A further experiment was performed to examine the hypothesis that the smaller subunit in the tetrameric form of prolyl hydroxylase is not synthesized directly, but must be derived from a precursor molecule such as cross-reacting protein. Since the assembly of the active prolyl hydroxylase was slow, chick tendon cells were grown in primary cultures and were labelled during the exponential phase of growth with [3 H]leucine. Since the cells were dividing, all forms of prolyl hydroxylase were labelled. The cells were chased and a first-order plot of the decay of radioactively labelled proteins was

Fig. 8. Chase of radioactive labelled α - and β -subunits and cross-reacting protein in cultured tendon fibroblasts

The cultured tendon fibroblasts at mid-exponential phase were labelled with [3 H]leucine for 4 h and the label was chased with unlabelled leucine for different periods of time as indicated. The percentages of radioactivity at the end of the labelling period in ($\square$) total protein, ($\bullet$) α - and ($\circ$) β -subunits and ($\triangle$) cross-reacting protein were plotted as a function of the chase time (see the Experimental section).

used to assess the turnover times for the α - and β -subunits of the active enzyme, of cross-reacting protein, and of total cellular protein. The results indicated that α -subunit, cross-reacting protein and total cellular protein turned over with half-lives of 60 h (Fig. 8). In contrast, the β -subunit increased for 20 h during the chase period, indicating that it was being formed from a labelled precursor. Because of the long chase period required, it was not possible to determine the turnover of labelled β -subunit after it acquired maximal labelling at 20 h, but it is likely that its half-life would be 60 h or more.

Discussion

It has been known for some time that fibroblasts, and indeed many non-fibroblast cells, contain an active form of prolyl hydroxylase and cross-reacting protein (McGee *et al.*, 1971; McGee & Udenfriend, 1972; Stassen *et al.*, 1974; Kuutti *et al.*, 1975; Kao *et al.*, 1975; Tuderman & Kivirikko, 1977; Chen-Kiang *et al.*, 1977; Risteli *et al.*, 1978). Here we report that cross-reacting protein represents at least in part a precursor of the β -subunit in the active tetrameric form of prolyl hydroxylase. When freshly isolated tendon cells were labelled for 4 h, the α -subunit and cross-reacting protein were labelled,

but the β -subunits in the newly assembled tetramers were unlabelled. When the label was chased for 4 h, no appreciable amount of label was chased into the β -subunit, indicating that the pool of cross-reacting protein must be quite large compared with the amount of tetramer formed in 4 h. The data presented here indicate that cross-reacting protein and β -subunit are not identical nor in equilibrium, and predict that cross-reacting protein must be processed in some way to be converted into β -subunit. The reason for an altered mobility of at least one peptide derived from cross-reacting protein compared with peptides derived from β -subunit has so far not been explored.

In order to demonstrate a precursor-product relationship between the cross-reacting protein and β -subunit, the turnover times of the α - and β -subunits and cross-reacting protein in cultured fibroblasts were determined. The results indicated that, as labelled cross-reacting protein decreased in cells, there was a corresponding increase in labelled β -subunit in the tetrameric form of prolyl hydroxylase. A long half-time was previously found for prolyl hydroxylase *in vivo* in rabbit skin (Chichester *et al.*, 1979) and in chick embryos (Majamaa *et al.*, 1979).

When the inactive form of prolyl hydroxylase was first observed in fibroblasts, experiments were performed to suggest that the enzyme could be activated under some circumstances in the absence of protein synthesis, implying that cross-reacting protein consisted of unassembled subunits from the active enzyme (Stassen *et al.*, 1974; Tuderman *et al.*, 1977). The results presented in several other reports, however, indicate that cross-reacting protein consists only of one polypeptide chain; therefore such an activation mechanism seems unlikely (Chen-Kiang *et al.*, 1977; Kao & Berg, 1979). The results presented here predict that, for prolyl hydroxylase to increase in fibroblasts, new protein synthesis of the α -subunit is required. This prediction has been substantiated in L-929 cells, where protein synthesis was shown to be required for an increase in enzyme activity when the cells were grown at higher density (Kao & Berg, 1979). Since cross-reacting protein was the only labelled protein in the cells that was both immunologically and structurally related to β -subunit (Figs. 1, 5–7), it is likely that cross-reacting protein serves as a precursor for β -subunit.

The data presented here demonstrate that at least one function of cross-reacting protein in fibroblasts is to serve as a precursor of β -subunits in the assembly of the tetrameric form of prolyl hydroxylase in fibroblasts. The assembly process may be similar to the assembly of lactose synthase, where α -lactalbumin is identical with B-protein of the enzyme (Brodbeck *et al.*, 1967) and associates with A-protein of the enzyme to form active lactose

synthase when more enzyme is needed for the synthesis of lactose (Brew *et al.*, 1968).

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