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CYSTEINYL-CYSTEINE AND THE MICROSOMAL PROTEIN FROM WHICH IT IS DERIVED ACT AS REDUCING COFACTOR FOR PROLYL HYDROXYLASE

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Summary: Microsomes from L-929 cells contain a reductant which can replace ascorbate as a cofactor for prolyl hydroxylase. The cofactor was extracted with Triton X-100 and exhibited high and low molecular weight forms on S-300 gel columns. Refiltration or trypsin treatment of high molecular weight cofactor produced additional low molecular weight form. The low molecular weight form was purified by P-2 gel filtration, and Dowex-1 and thin layer chromatography. It is ninhydrin reactive, exhibits reduced and oxidized forms with molecular weights of 240 and 460, respectively, and yielded cystine upon acid hydrolysis. The results suggest that it is a dipeptide, cysteinyl-cysteine, derived from a microsomal protein which is the high molecular weight cofactor.

Prolyl hydroxylase (prolyl-glycylpeptide, 2-oxoglutarate:oxygen oxidoreductase, 4-hydroxylating,EC 1.14.11.2), which carries out an important post-translational step in collagen biosynthesis, requires a reductant in addition to α-ketoglutarate, Fe^{+2}, and oxygen (1). Ascorbate is the most efficient reductant and appears to act by reducing Fe^{+3}, free or enzyme-bound (2,3). Several substances, however, such as DTT, tetrahydropteridine and cysteine can replace ascorbate to varying extents (2,4). In stationary phase L-929 cells which cannot synthesize ascorbate, almost complete hydroxylation of proline occurs in the absence of ascorbate (5). We suggested that ascorbate-independent proline hydroxylation was due to an endogenous cofactor (5,6) and showed that L-929 cell extracts contained a reductant which could replace ascorbate for cell-free proline hydroxylation (4) and which was localized in membranes of isolated microsomes (7). This report describes the characterization of the cofactor activity.

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Abbreviations:

DTT,dithiothreitol; HMW,high molecular weight; LMW,low molecular weight; TLC, thin layer chromatography.

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Materials and Methods

The sources of most chemicals and radioisotopes have been described previously (4,7). Gly-gly-cys and cys-gly were purchased from Vega Biochemicals, Tucson, AZ and Avicel cellulose thin layer plates were from Analtech Inc, Newark, DL.

L-929 cells were grown in spinner culture using modified Eagle's minimum essential medium (4) with 10% fetal calf serum but without calcium and ascorbate. Cells (5×10^9) were harvested at maximum density by centrifugation, the pellet was resuspended in 10 mM Tris-HCl, pH 7.6/150 mM KCl (buffer A) and centrifuged again. This process was repeated and the volume of the cell pellet was measured. Cells were sonicated in an equal volume of buffer A, GSH was added to give a final concentration of 1.5 mM and the sonicate was centrifuged at 43,500 x g for 20 min. The pellet was extracted for 20 min at 0° with a volume of buffer A/1.5 mM GSH/1% Triton X-100 equal to the original sonicate volume and the suspension was centrifuged at 43,500 x g. The clear extract was removed and the pellet was reextracted with one-tenth as much buffer. The two extracts were combined and detergent was removed by stirring with Biobeads SM2 (0.3g/ml) for 2 h at 4°(8). The detergent-free extract was lyophilized, taken up in water to give a 10-fold concentration and insoluble material was removed by centrifugation.

Gel filtration: Initially the detergent-free extract was fractionated on a Sephacryl S-300 column (1.2 x 17cm) equilibrated and eluted with 50 mM Tris-HCl, pH 7.6/75 mM KCl/1.5mM GSH (1.5 ml fractions). In later preparations, a P-10 column (0.7 x12 cm) was used to separate high and low molecular weight forms of cofactor. Equilibration and elution were carried out with 10 mM Tris, pH 7.6/1.5mM GSH. Fractions(0.5 ml) were assayed for cofactor activity. Active fractions were pooled, lyophilized, taken up in 1 ml H_2O, and low molecular weight cofactor was further fractionated on a Bio-Gel P-2 column (1 x 20 or 40cm).It was equilibrated and eluted with 10 mM Tris-HCl, pH 7.6 with or without 1.5 mM GSH or 0.1 mM DTT, as indicated in the text or figure legends. Fractions(0.6 ml) were assayed for reducing cofactor and active fractions were pooled, lyophilized and taken up in H_2O equal to one-sixth the volume.

Purification of Low Molecular Weight (LMW) Cofactor: The peak of LMW cofactor activity was applied to an H_2O-washed, 1.5 ml column of Bio-Rad AG 1-X8 (Cl^-) and it was eluted with 3 ml of H_2O. The sample was lyophilized and further purified by thin layer chromatography, as described in the text. Ninhydrin-reactive material was detected with a 0.3% spray (in 95% ethanol) on plates or by assay of solutions(9).

Assays: Reducing cofactor was measured using a modified prolyl hydroxylase assay (4). NADPH (0.75 mM) was added to reduce the oxidized form of the cofactor (4,7) unless indicated otherwise. Results are expressed either as cpm ^3H_2O released or as nmoles, calculated on the basis of ascorbate standards (4). Cytochrome C-NADPH reductase and its flavoprotein component, ferricyanide-NADPH reductase, were assayed as previously described (10), using 0.02 mM NADPH. Protein was assayed by a dye-binding procedure (11) using Bio-Rad reagent.

Results and Discussion

Triton X-100 solubilized 90-100% of the endogenous prolyl hydroxylase cofactor in the particulate fraction of L-929 cell sonicates. When detergent-free extract was applied to a Sephacryl S-300 column, two peaks of cofactor activity

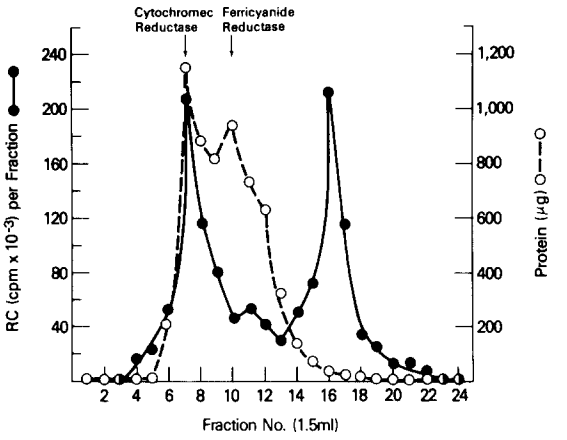
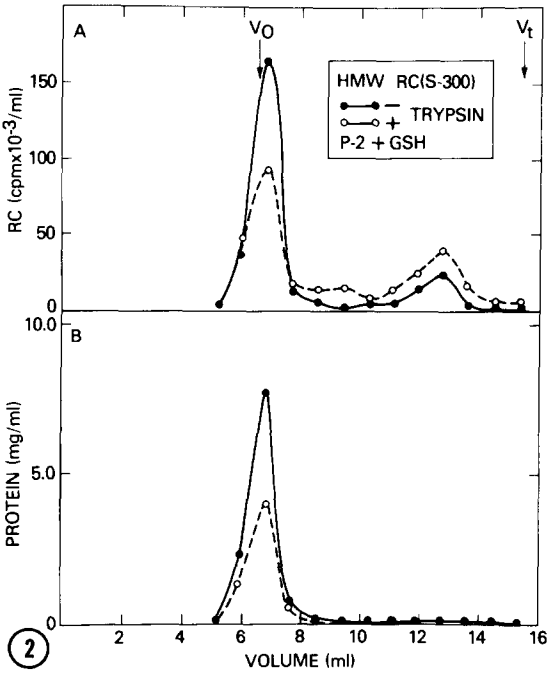


Fig.1. S-300 filtration of detergent-free extract. Filtration and analyses for protein, cofactor (RC) and enzymes were carried out as described in Methods. Fractions 6-9(Vo) contained HMW and 15-18 contained LMW cofactor.

were observed: a high molecular weight (HMW) form eluting at the V_o and the LMW form (Fig. 1). Because cofactor activity can be stimulated by NADPH, we measured the activity of NADPH-cytochrome C reductase and its flavoprotein component, NADPH-ferricyanide reductase. The first of these activities also occurred in the V_o fraction but the cofactor activity was clearly distinct from the second activity, making it unlikely that it was related to the cytochrome C reductase system.

The HMW cofactor appears to be a protein, since trypsin treatment decreased its activity with a concommitant conversion of 49% of the activity to a LMW form (Fig. 2A) and a decrease in total protein similar to the decrease in HMW cofactor activity (Fig. 2B). Even without trypsin treatment, 21% of the HMW activity was converted to the LMW form (Fig.2A, solid line) and in other experiments there was was as much as 50% conversion upon refiltration, with no loss of total activity (data not shown). The LMW form obtained by refiltration exhibited the same properties as the purified cofactor initially derived from S-300, but we have not determined whether the trypsin-generated material is identical.

The LMW cofactor from S-300 showed a single peak of activity on P-2 in the absence of GSH (Fig.3A). When this peak was rerun on a longer column without GSH a single peak was again obtained (Fig.3B), but with GSH an additional peak of



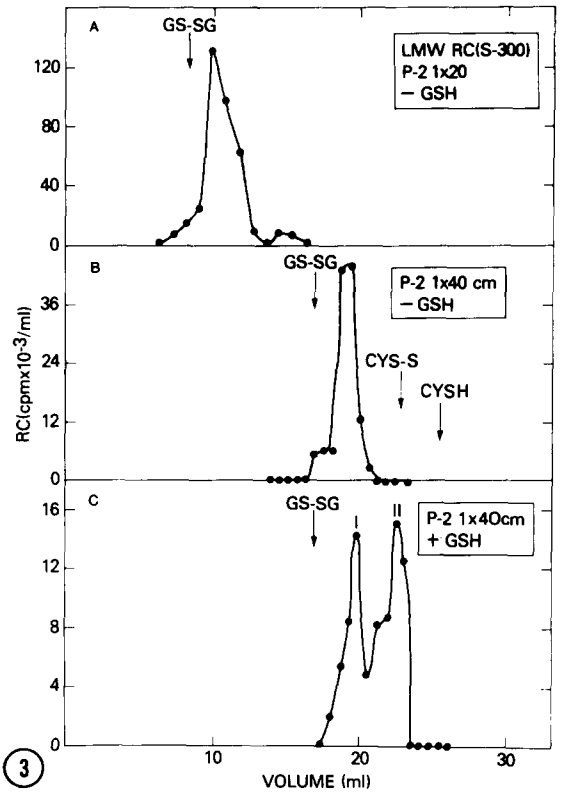


Fig.2. Effect of trypsin on HMW cofactor. HMW cofactor from an S-300 column was incubated with (o--o) or without (●—●) 0.29 mg/ml of trypsin for 15 min at 24° and then an equal amount of soybean trypsin inhibitor was added. The samples were applied to a P-2 column with GSH and fractions were assayed for A., cofactor (RC) activity and B., protein concentration, as described in Methods.

Fig.3. Oxidized and reduced forms of LMW cofactor. LMW cofactor from an S-300 column was applied to a P-2 column (1 x 20 cm) without GSH (A). The peak of cofactor (RC) activity was pooled, lyophilized and portions were applied to a 1 x 40cm column in the absence (B) or presence (C) of GSH. Molecular weight markers were GS-SG, cystine (CYS-S) and cysteine (CYSH).

activity (II) appeared with approximately one-half the molecular weight of the first peak (I) (Fig.3C). This behavior suggested that the molecule contained a sulfhydryl group, presumably cysteine, and that the lower molecular weight peak (II) corresponded to a reduced form, while the oxidized form (I) was a dimer. With DTT (0.1mM) in the elution buffer, most of the cofactor was in the reduced form (data not shown) and it was used for routine purification on P-2.

The peak of reduced LMW cofactor obtained from P-2 columns contained a large amount of ninhydrin-reactive and UV absorbing material. The latter could be

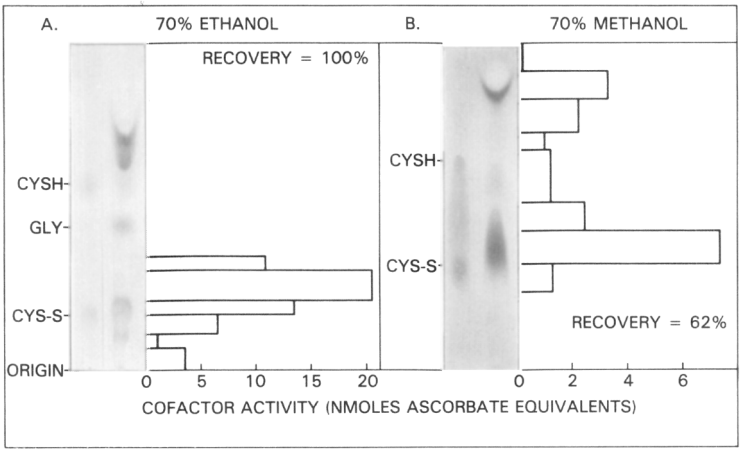


Fig.4. Preparative TLC. LMW cofactor from the AG 1 step was streaked or spotted on a TLC plate for recovery of activity or detection with ninhydrin, and the plate was developed in 70% ethanol (A). Sections were scraped off and extracted with 50% methanol. Solvent was evaporated, samples were assayed for cofactor and the three most active fractions were pooled and evaporated. The sample was rechromatograhped (B) in 70% methanol using the same procedures, but only the most active fraction was retained. The first lane in each case is a cysteine marker which contains reduced (CYSH) and oxidized (CYS-S) forms.

removed by passing the sample over an AG 1 column. Ninhydrin-reacting material, which proved to be mainly free amino acids, could be removed by TLC. The AG 1-treated sample was chromatographed twice on cellulose plates in 70% ethanol. Results of the second run are shown in Fig. 4A. A peak of cofactor activity was observed which corresponded to a faint ninhydrin-positive spot migrating slightly faster than cystine. The peak fraction and the two adjoining fractions were pooled and chromatographed in 70% methanol, which increased the mobility of the cofactor (Fig.4B). Again, the majority of the cofactor activity migrated just ahead of cystine and corresponded to an intense ninhydrin-positive spot, indicating a substantial purification. The single peak fraction was analyzed further.

Since the cofactor appeared to contain cysteine, yet the molecular weight of its reduced form was twice that of cysteine, we considered the possibility that the cofactor was a peptide. GSH does not exhibit cofactor activity (4,7) but two peptides containing cysteine, gly-gly-cys and cys-gly, were tested and found to be active and, like the endogenous cofactor, they required NADPH for maximum activity. In contrast, the activity of cysteine was not affected by NADPH (4) and cystine

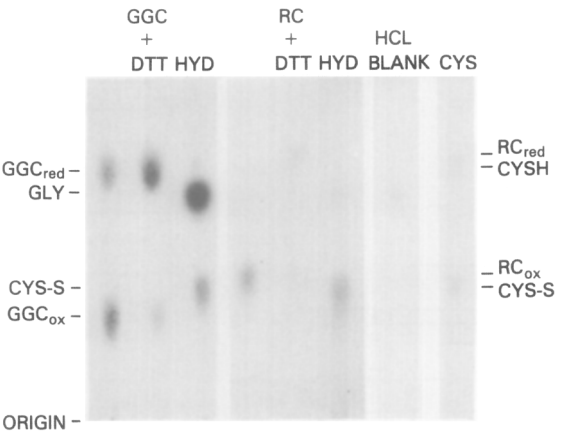


Fig.5. Analysis of LMW cofactor (RC). Purified LMW cofactor samples were applied to TLC after reduction with 10 mM DTT or hydrolysis with 6 N HCl (30 min,150°,under N_2). Gly-gly-cys(GGC, 10 nmoles) was treated identically. A hydrolysis blank with HCl but no sample was used to identify impurities in reagents. Partially oxidized cysteine was used as a marker (cys), as described in the legend to Fig. 4 oxidized (ox) and reduced (red) forms of peptides are indicated.

was inactive (data not shown). Since these results supported the possibility of a peptide cofactor, a portion of purified cofactor was acid hydrolyzed prior to TLC- and DTT was added to another portion in order to locate the reduced form. Gly-gly cys was treated identically to check on completeness of hydrolysis and reduction. It showed two spots prior to treatment with DTT (Fig. 5) and after treatment there was an increase in the intensity of the upper spot, indicating it was the reduced form, although reduction was not complete. The purified cofactor showed a single ninhydrin positive spot migrating slightly ahead of cystine, in agreement with the preparative runs. Reduction produced an additional spot migrating slightly ahead of cysteine. Hydrolysis conditions were adequate as judged by the complete release of gly and cystine from gly-gly-cys. Hydrolysis of the purified cofactor produced only cystine. A faint spot migrating like gly was considered to be derived from an impurity in the reagents, since it also was present in the HCl blank.

These results and the occurrence of reduced and oxidized forms with molecular weights about twice that of cysteine and cystine, respectively, suggest that the reduced form of the LMW cofactor is a dipeptide, cysteinyl-cysteine, and that the

oxidized form is a disulfide-bonded dimer. We further propose that the reducing cofactor for prolyl hydroxylase in L-929 cells is a protein in the membrane of the rough endoplasmic reticulum with a cys-cys sequence which can become fully reduced under certain metabolic conditions, as in stationary phase cultures (4). This sequence may be terminally situated, allowing access into the cisternae where prolyl hydroxylase and emerging procollagen chains are located. A terminal location of cysteine may in fact be required for activity, based on the observations that GSH, with an internal cysteine, is inactive while cys-gly and gly-gly-cys are active. The accessibility of this region of the microsomal protein, the presumed HMW cofactor, also may make it susceptible to proteases released from contaminating lysosomes (4) during extraction, thus accounting for formation of the LMW cofactor.

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