# IN VIVO LABELING AND TURNOVER OF PROLYL HYDROXYLASE AND A RELATED IMMUNOREACTIVE PROTEIN

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SUMMARY: Prolyl hydroxylase and an immunologically related protein (CRP) were purified from neonatal rabbit skin at various time periods following administration of $^{3}$H-leucine. The peak incorporation of label into prolyl hydroxylase was found to be 12 hours, while peak incorporation into CRP occurred within 2 hours. Semi-log plots of the loss of radioactivity from these protein pools against time indicated an apparent $T_{\frac{1}{2}}$ for prolyl hydroxylase of 78 hours, and a $T_{\frac{1}{2}}$ of CRP of 44 hours. Calculated $K_d$ values indicate that that breakdown of active enzyme does not account for the amount of CRP found in tissues.

Prolyl hydroxylase (EC 1.14.11.2; proline, 2-oxoglutarate dioxygenase) is a mixed function oxidase which converts specific prolyl residues in peptide precursors of collagen to 4-hydroxyproline. Prolyl hydroxylation is thought to be one of the critical cellular events necessary for the synthesis and secretion of structural collagen (1). Although the importance of prolyl hydroxylase as a controlling factor in collagen synthesis is unclear, large increases in activity have been reported in a variety of tissues responding to injury induced damage which result in increased collagen synthesis (2). This parallelism has led to the use of prolyl hydroxylase as a marker for determining changes in the rate of collagen synthesis, and has helped generate interest in the cellular regulation of this hydroxylase.

McGee, et al. (3) obtained evidence with antibody against rat skin prolyl hydroxylase that an enzymatically inactive antigen is present in L-929 fibroblasts. Examination of other animal tissues using antibody directed against rat (4), rabbit (5) or human prolyl hydroxylase (6) have confirmed the presence of both forms of antigen (active enzyme and immunologically

cross-reacting protein [CRP]) in all other mammalian tissues with CRP always present in excess relative to active hydroxylase. The significance of CRP has been under investigation for some time (4) and some of its properties have been ascertained (3) (4) (6). The available data support the conclusion that CRP is structurally related to prolyl hydroxylase. However, the question still remains whether CRP is a precursor or a degradation product of prolyl hydroxylase. In this study we have examined the relationship between enzyme and CRP through determination of the relative turnover of each in rabbit skin.

## MATERIALS AND METHODS

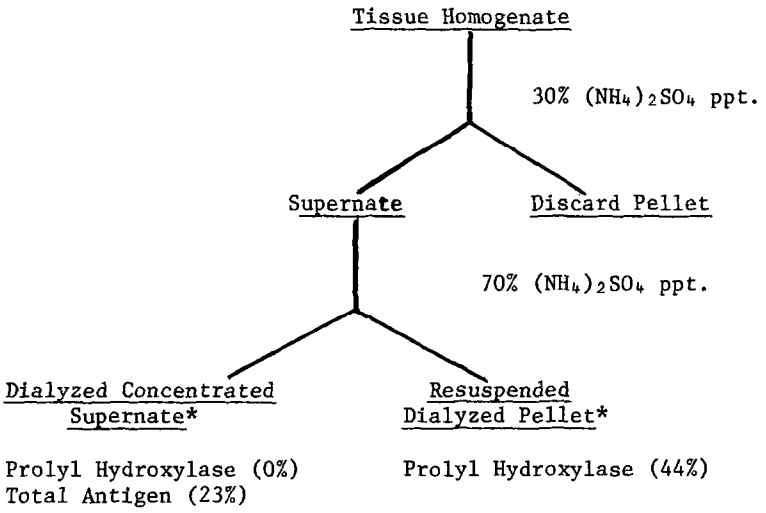
Prolyl hydroxylase was measured by the tritium release assay (7) and prolyl hydroxylase related antigen or CRP was measured by the enzyme immunoassay of Stassen et al. (4) using antibody to rabbit prolyl hydroxylase as previously described (5). Protein was determined by the method of Lowry et al. (8).

Three day old rabbit pups were injected with 5 mc/60g of 4-5 $^{3}$H-leucine interperitoneally (42.6 Ci/m mole New England Nuclear) and returned to their mother until killed. Animals were killed at various time periods, the skins quickly removed and homogenized 1:5 in cold 0.25 M sucrose containing $10^{-5}$M dithiothreitol (DTT), $10^{-5}$M and ethylenediaminetetraacetic acid (EDTA). The homogenate was centrifuged at 20,000 x g for 30 min and the supernate was brought to 30% saturation in $(NH_4)_2SO_4$ (via addition of saturated $(NH_4)_2SO_4$ solution). After centrifugation, the pellet was removed, the supernate was brought to 70% $(NH_4)_2SO_4$ saturation, and the precipitated protein was collected by centrifugation at 45,000 x g for 2 hours. The precipitate was dissolved in 0.05 M Tris (pH 7.4) containing 0.2 M glycine, 0.2 M NaCl, and $10^{-5}$M in EDTA and DTT and dialyzed against the same buffer. After centrifugation at 45,000 x g this enzyme solution was placed on affinity columns consisting of reduced and carboxymethylated ascaris collagen coupled to Sepharose 4B as previously described (9). Each sample was applied to an in-

TABLE 1

PERCENT RECOVERY OF PROLYL HYDROXYLASE AND

TOTAL ANTIGEN DURING PURIFICATION




*These fractions were processed as described in the text.

dividual 1.5 x 4 cm column, and enzyme was eluted with 1 ml of buffer containing 10 mg/ml (pro-gly-pro) (M.W.2300). After concentration and washing by ultrafiltration (MINICON$^R$-A-25) enzyme activity and the $^3H$ content were determined.

CRP was purified from the 70% $(NH_4)_2SO_4$ supernate after dialysis against 0.05 M Tris (pH 7.4) buffer containing 0.1 M NaCl, and $10^{-5}$M in EDTA and DTT, and concentrated in an ultrafiltration cell (AMICON$^R$-PM-30 membrane). Each sample was adjusted in volume to a uniform concentration of CRP and antibody was added under optimal conditions for maximum precipitation of CRP-antibody complex. This precipitate was collected and washed twice by centrifugation, dissolved in 1 N NaOH, and collected on filters (Whatman GF/A) in the presence of 10% trichloroacetic acid containing $10^{-3}$M leucine. The fil-

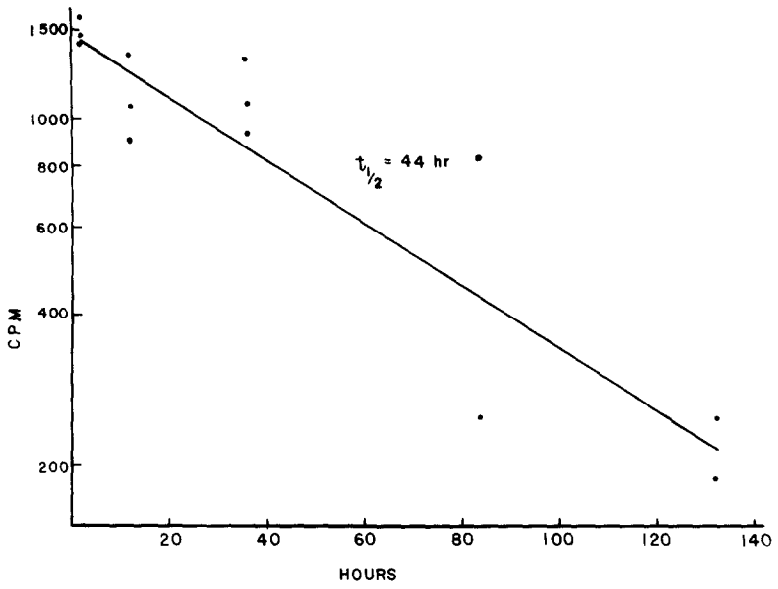



FIGURE 1 - Loss of Label from Prolyl Hydroxylase v.s. time (Best fit line from computer regression program).

ters were washed, digested in Protosol$^{R}$ (New England Nuclear) in scintillation vials and the $^{3}H$ content determined.

## RESULTS AND DISCUSSION

The recovery of total antigen and prolyl hydroxylase during the purification of the enzyme and CRP are shown in Table 1. The 70% $(NH_4)_2SO_4$ precipitation step completely removed prolyl hydroxylase activity from the supernate so that during the antibody precipitation we were dealing with a CRP fraction that was devoid of active enzyme. The pellet from the 70% precipitation did contain some CRP, however this does not bind to the affinity column and thus would not contaminate the final enzyme preparations. This point was confirmed by gel electrophoresis. The fractionation of CRP by this technique leaves open a possibility that a component of a heterogenous protein pool is lost from the turnover study.

Figure I shows the decay of radioactivity in prolyl hydroxylase as a function of time. Assuming random decay of prolyl hydroxylase molecules,

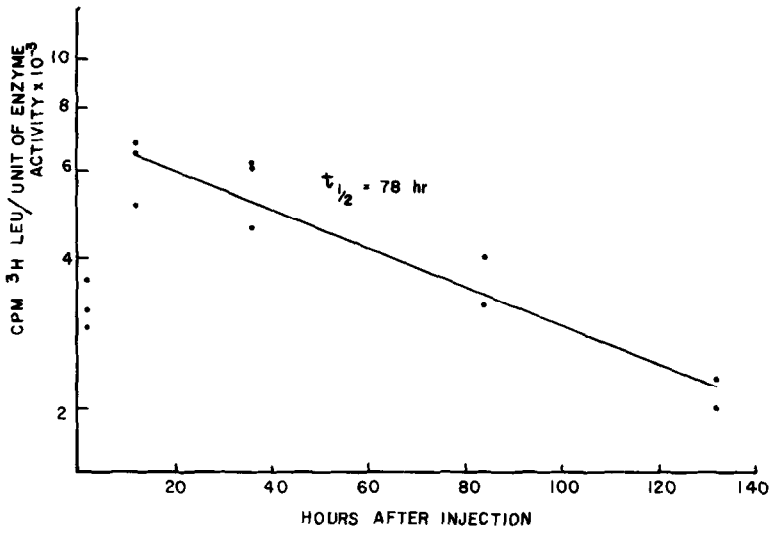



FIGURE 2 - Loss of Label from CRP v.s. time (Best fit line from computer regression program). CRP obtained as described in text.

the points were regressed by computer and the line representing a $T_{\frac{1}{2}}$ of 78 hours was plotted (significant at the $P<.005$ level). Thus, prolyl hydroxylase is an enzyme with a relatively slow apparent turnover rate, which might explain *in vivo* data showing that prolyl hydroxylase increases over a several day period following injury (10). This would also suggest new synthesis rather than activation as a means of elevating prolyl hydroxylase levels in fibrotic states. Figure I also shows that maximum label incorporation does not occur until approximately 12 hours after injection. Other studies we have completed indicate this peak occurs 8 to 12 hours after administration of label.

Figure II shows the decay of radioactivity in immunoprecipitates from the 70% supernatant fraction. These precipitates were analyzed on 10% SDS gels and the radioactivity was shown to be contained in an area of the gel that corresponded to the two major subunits of prolyl hydroxylase (11). From the regression line the $T_{\frac{1}{2}}$ of CRP is 44 hours. There was no detectable delay in label incorporation into CRP (peak occurred within 2 hours). It

has been shown that in proteins with long half lives it takes a significant amount of time (on the order of hours) to reach maximum labeling (12). The difference between prolyl hydroxylase and CRP in the time required to reach maximum label incorporation may reflect a relationship between these two proteins or may be a result of the difference in their turnover rates. The influence of $^{3}$H-leucine reutilization on the $T_{\frac{1}{2}}$ values reported here is being examined at the present time. Since neonates were used in this investigation the decay curves reflect both turnover and dilution due to tissue growth. However, inferences about the relationship between prolyl hydroxylase and CRP remain valid since both decay curves are subject to the same dilutional effect.

We found that prolyl hydroxylase activity of neonatal skin did not change significantly over the labeling period reported. Prolyl hydroxylase activity was determined to be 81.1±7.30 cpm/μg protein (±S.D.) as assayed in the 20,000 x g skin supernatants. CRP, measured in the same enzyme units, was 320.4±21.9 cpm/μg protein while total antigen was 412.2±20.9 cpm/μg protein. Thus in these studies, as in earlier reports (4) (6), CRP levels were much higher than prolyl hydroxylase levels. From the $T_{\frac{1}{2}}$ values the rates of degradation (Kd) of CRP and prolyl hydroxylase were determined to be .0158 $hr^{-1}$ and .0089 $hr^{-1}$ respectively. Multiplying the rate of degradation by the level in neonatal skin we can estimate the proportion of prolyl hydroxylase and CRP molecules being broken down per unit of time. These values would indicate that all of the CRP in tissue cannot be accounted for by the breakdown of prolyl hydroxylase. Thus these data would support the hypothesis that a portion of the CRP pool in cells is precursor to active enzyme.

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