Effect of Hepatic Injury on Prolyl 3-Hydroxylase and 4-Hydroxylase Activities in Rat Liver and on Immunoreactive Prolyl 4-Hydroxylase Concentrations in the Liver and Serum

By JUHA RISTELI, LEENA TUDERMAN, KARL TRYGGVASON and KARI I. KIVIRIKKO

Department of Medical Biochemistry, University of Oulu, Oulu, Finland

(Received 13 June 1977)

After severe hepatic injury induced by dimethylnitrosamine, approximately a 4-fold increase in hepatic prolyl 4-hydroxylase activity occurred within 4 days, whereas the increases in total immunoreactive prolyl 4-hydroxylase protein and in prolyl 3-hydroxylase activity were only about 1.4-fold. The different magnitudes of the increases in the prolyl 4-hydroxylase and 3-hydroxylase activities were verified after partial purification of the enzymes by gel filtration. The data support previous reports indicating differential increases in the activities of individual enzymes of collagen biosynthesis in hepatic injury. Separation of the prolyl 4-hydroxylase tetramers from the monomer-size protein by gel filtration indicated that the increase in enzyme activity was similar to that in enzyme tetramers, and an increase had also occurred in the ratio of enzyme tetramers to total enzyme protein. Thus the specific activity of the tetramers had remained unchanged in liver injury. The administration of dimethylnitrosamine was also accompanied by a marked increase in the immunoreactive prolyl 4-hydroxylase protein concentration in the serum, and a similar effect was also noted after carbon tetrachloride administration, results suggesting that the increases originated in the liver.

Collagens have a high hydroxyproline content, this imino acid being found in only a few other vertebrate proteins. Most of it is present in the form of the trans-4-isomer, but all collagens also contain some trans-3-hydroxyproline (for reviews, see Fietzek & Kühn, 1976; Piez, 1976). The synthesis of the two hydroxyprolines is catalysed by separate enzymes, prolyl 4-hydroxylase and prolyl 3-hydroxylase (Risteli et al., 1977; Tryggvason et al., 1977). Hepatic injury is associated with an early increase in prolyl 4-hydroxylase activity (see Risteli & Kivirikko, 1974, 1976), whereas nothing is known about possible changes in prolyl 3-hydroxylase activity. In the present work an attempt was made to measure prolyl 3-hydroxylase activity in the liver and to study whether changes in this enzyme activity in liver injury are similar to those of prolyl 4-hydroxylase activity.

The initial rise in prolyl 4-hydroxylase activity in liver injury is not associated with any increase in total immunoreactive prolyl 4-hydroxylase protein (Risteli et al., 1976). This enzyme protein is present in isolated cells (McGee & Udenfriend, 1972; Stassen et al., 1973; Kao et al., 1975) and intact tissues (Stassen et al., 1974; Tuderman, 1976; Tuderman & Kivirikko, 1977) in two forms, the active enzyme tetramers and an inactive form the size of which corresponds to that of the enzyme monomers. In several instances increases in prolyl 4-hydroxylase activity have been found to be associated with an increase in the ratio of active enzyme tetramers to total enzyme protein, so that the apparent specific activity of the tetramers has remained unchanged (Kao et al., 1975; Tuderman, 1976; Tuderman & Kivirikko, 1977). On the other hand, the rise in prolyl 4-hydroxylase activity in the liver in experimental atherosclerosis has been attributed to an increase in the specific activity of these enzyme tetramers (Fuller et al., 1976). We therefore studied whether the increase in prolyl 4-hydroxylase activity in hepatic injury is primarily due to an increase in the concentration of enzyme tetramers or an increase in their specific activity.

The presence of prolyl 4-hydroxylase activity has also been reported in rat and human serum (Stein et al., 1970; Keiser et al., 1972, 1975; Langness et al., 1975), although it is relatively low and has failed to be detected in some studies (Takeuchi & Prockop, 1969). This activity increases in certain conditions affecting the liver, such as experimental liver fibrosis (Langness et al., 1975) and hepatocellular carcinoma (Keiser et al., 1972, 1975). It is not known whether the increases in serum prolyl 4-hydroxylase activity are due to an increase in the amount of enzyme protein, a decrease in the presence of inhibitors in
the serum (Stassen et al., 1974) or an alteration in the ratio of active to inactive enzyme. The present work investigates whether the liver injury is accompanied by any changes in serum prolyl 4-hydroxylase protein.

Experimental

Animals and preparation of samples for assays

The experimental animals were female Sprague–Dawley rats, aged 2.5 months at the beginning of the experiments. They were fed on a commercial diet (Hankkija Oy, Helsinki, Finland) and allowed free access to water. Severe liver injury was induced by administering dimethyl nitrosamine intraperitoneally at a dose of 5 µl (diluted 1:25 with 0.15 M-NaCl)/100g body wt. The six rats treated in this manner were then killed in groups of two on days 2, 3 and 4 after the injection, a further two rats killed on day 2 serving as controls. The rats were killed by anaesthesia with diethyl ether and their livers rapidly removed and stored at −70°C until assayed. The livers were homogenized in a Teflon/glass homogenizer (Thomas, Philadelphia, PA, U.S.A.) at about 1500 rev./min for 60s in a cold (0°C) solution consisting of 0.2 M-NaCl, 0.1 M-glycine, 50 µM-dithiothreitol, 0.1% (w/v) Triton X-100, 0.01% (w/v) soya-bean trypsin inhibitor (Sigma Chemical Co., Kingston-upon-Thames, Surrey, U.K.) and 20 mM-Tris/HCl buffer, adjusted to pH7.5 at 4°C (Risteli & Kivirikko, 1974, 1976; Risteli et al., 1977). The volume of the solution was 9 ml/g of liver. The homogenates were incubated at 4°C for 30 min and then centrifuged at 15000g for 30 min at 4°C. Portions of the supernatant were then used for the assay of the enzyme activities, prolyl 4-hydroxylase protein and the supernatant protein.

To study the effect of acute liver injury on the serum immunoreactive prolyl 4-hydroxylase concentration, a further 15 rats divided into three groups were given dimethylnitrosamine intraperitoneally in doses of 1 µl, 5 µl or 10 µl (diluted 1:100 or 1:25 with 0.15 M-NaCl)/100g body wt., a fourth group of six rats serving as controls. In another experiment liver injury was induced by injecting carbon tetrachloride subcutaneously at a dose of 100 µl (diluted with an equal volume of paraffin oil)/100g body wt. In this case the control group and the liver-injury group each contained six rats. The blood samples were taken from the abdominal aorta by operation under ether anaesthesia, and the sera stored in a frozen state until assayed.

Assays of prolyl 4-hydroxylase activity and protein

Portions of the 15000g supernatants or gel-filtration fractions were incubated with agitation for 30 min at 37°C in a final volume of 2 ml containing 50000 d.p.m. of [14C]proline-labelled protocollagen substrate, 0.08 mM-FeSO₄, 2 mM-ascorbic acid, 0.5 mM-2-oxoglutarate, 0.2 mg of catalase (Calbiochem, Hereford HR4 9BR, U.K.)/ml, 0.1 mM-dithiothreitol (Calbiochem), 2 mg of bovine serum albumin (Sigma)/ml and 50 mM-Tris/HCl buffer, adjusted to pH7.8 at 25°C (see Risteli & Kivirikko, 1974). The reaction was stopped by adding an equal volume of conc. HCl, and the amount of hydroxy-[14C]proline formed assayed after hydrolysis at 120°C overnight (Juva & Prockop, 1966). The [14C]-proline-labelled protocollagen substrate was prepared as described previously (Risteli et al., 1976).

Prolyl 4-hydroxylase protein was measured by a direct radioimmunoassay based on the inhibition by the non-labelled enzyme of complexing of the radiolabelled enzyme by antibody and subsequent precipitation of the enzyme–antibody complex by a cellulose-bound second antibody (Tuderman et al., 1975b). Prolyl 4-hydroxylase was purified from newborn rats by affinity chromatography by using poly-L-proline (Tuderman et al., 1975a; Risteli et al., 1976). The enzyme was entirely pure when examined by polyacrylamide-gel electrophoresis as a native protein or in the presence of sodium dodecyl sulphate (Risteli et al., 1976). Part of this preparation was labelled with 3H by using a technique of reductive alkylation with formaldehyde and NaBH₄ (see Tuderman et al., 1975b). Antiserum to rat prolyl 4-hydroxylase was prepared in rabbits as described previously (Risteli et al., 1976). The assays in serum were carried out with 20 µl of serum and the results processed and converted into µg of immunoreactive prolyl 4-hydroxylase on a Honeywell time-sharing system by a modification of a program reported for radioimmunoassays (Burger et al., 1972).

Assay of prolyl 3-hydroxylase activity

To assay the prolyl 3-hydroxylase activity, portions of the 15000g supernatants or gel-filtration fractions were incubated with agitation for 40 min at 20°C in a final volume of 2 ml containing 1000000 d.p.m. of [2,3-3H]proline-labelled fully 4-hydroxylated protocollagen substrate and other compounds as described above for the assay of prolyl 4-hydroxylase activity (Risteli et al., 1978). The reaction was stopped by adding 0.5 ml of 10% (w/v) trichloroacetic acid, and the 3H₂O formed during 3-hydroxylation assayed by vacuum distillation of the whole reaction mixture (see Hutton et al., 1966). The [2,3-3H]proline-labelled protocollagen substrate was prepared as described previously for [14C]proline (Risteli et al., 1976), and incubated with 5 µg of pure chick prolyl 4-hydroxylase (Tuderman et al., 1975a) for 4 h at 37°C in a final volume of 10 ml containing 0.08 mM-FeSO₄, 2 mM-ascorbic acid, 0.5 mM-2-oxoglutarate and 50 mM-Tris/HCl buffer, adjusted to pH7.8 at 25°C, to
convert all appropriate prolyl residues into 4-hydroxyprolyl residues (see Tryggvason et al., 1977). The solution was dialysed against 0.2 M NaCl/50 mM Tris/HCl buffer adjusted to pH 7.8 at 4°C, and the preparation stored frozen in batches of 1 ml containing 10^4 d.p.m. The substrate was heated at 100°C for 10 min immediately before use.

Other assays

The protein content of the 15000g supernatant of liver homogenates was assayed by the method of Lowry et al. (1951), with bovine serum albumin as standard.

All radioactivity counting was performed in a Wallac liquid-scintillation spectrometer with an efficiency of 85% and a background of 25 c.p.m. for ^14C radioactivity, or 35% and 10 c.p.m. for ^3H radioactivity. In the assay of prolyl 4-hydroxylase activity and protein the scintillants reported for these procedures were used (see Juva & Prockop, 1966; Tuderman et al., 1975a,b). In the assay of prolyl 3-hydroxylase activity a 2.0 ml sample of the ^3H_2O was dissolved in 5 ml of Instagel (Packard, Caversham, Berks., U.K.) and counted for radioactivity as above.

Results

Assay of prolyl 3-hydroxylase activity in the liver

A rapid assay has been developed for prolyl 3-hydroxylase activity (Risteli et al., 1978), based on the release of ^3H_2O from [2,3-^3H]proline-labelled polypeptide substrate which is fully hydroxylated with pure prolyl 4-hydroxylase (Tryggvason et al., 1977) but contains no 3-hydroxyproline. This assay was used in the present study. The standard curve prepared with increasing amounts of the 15000g supernatant of the liver homogenate was almost linear until about 900 d.p.m. was observed with about 0.5 ml of the supernatant added (Fig. 1). Thus all assays were carried out by using amounts that did not cause either of these limits to be exceeded. The experiment in which the prolyl 3-hydroxylase activity was studied after gel filtration served to verify the reliability of this assay for liver samples (see below).

Effect of hepatic injury on prolyl 4-hydroxylase activity and protein and on prolyl 3-hydroxylase activity

After severe hepatic injury induced with dimethyl-nitrosamine (5 ml/100 g body wt.) approximately a 4-fold increase occurred in hepatic prolyl 4-hydroxylase activity within 4 days (Fig. 2), but the increases in immunoreactive prolyl 4-hydroxylase protein and prolyl 3-hydroxylase activity were only about 1.4-fold. In fact, an initial decrease occurred in both of the latter (Fig. 2). Only two rats were killed in the treated groups at each time point, but owing to the marked differences between prolyl 4-hydroxylase activity and the other two properties measured, it seems obvious that the increase in prolyl 4-hydroxylase activity was much larger than that in the other two properties.

As it seemed possible that the liver might contain inhibitors that would partly mask the increase in prolyl 3-hydroxylase activity, prolyl 4-hydroxylase
and 3-hydroxylase activities were determined after partial purification of the enzymes by gel filtration (Tryggvason et al., 1977). In the sample from the injured liver applied to the gel-filtration column the prolyl 4-hydroxylase activity was 4.3 times and the 3-hydroxylase activity 1.4 times that of a control sample applied to a similar column. The sum of the enzyme activities in the gel-filtration fractions revealed a 5.5-fold increase in the 4-hydroxylase activity and one of 1.8-fold in the 3-hydroxylase activity (Fig. 3). These results suggest that the liver extract did not contain any appreciable amounts of inhibitors of these enzyme activities, unless the inhibitors were eluted in exactly the same fractions as the two enzymes, which is unlikely, or unless they remained tightly bound to the enzymes.

**Relationship between the increase in prolyl 4-hydroxylase activity and the amount of enzyme tetramers**

To study whether the distribution of the enzyme protein between the active tetramers and the inactive monomer-size protein altered in hepatic injury, a sample from the injured liver and a control sample were applied to similar gel-filtration columns. The enzyme activity in the former sample was about 4.3 times that in the latter. The amount of total immunoreactive enzyme protein in the gel-filtration fractions of the sample from the injured liver was increased about 1.5-fold, whereas that of enzyme tetramers had increased about 5.5-fold (Fig. 4). Thus the enzyme tetramers comprised about 16.0% of the total immunoreactive protein in the injured liver as contrasted with about 4.2% in the control liver.

---

**Fig. 3. Activities of prolyl 3-hydroxylase and 4-hydroxylase in gel-filtration fractions from one control (a) and one injured (b) rat liver**

Samples (2.5 ml) of the 15000g supernatants of homogenates from the control and injured livers (5 ml of solution/g of liver) were applied simultaneously to two similar columns (1.5 cm × 90 cm) of agarose A-1.5 m (Bio-Gel, 200–400 mesh; Bio-Rad, Bromley, Kent, U.K.). The columns were equilibrated and eluted with a solution containing 0.1 M NaCl, 0.1 M glycine and 20 m M Tris/HCl buffer adjusted to pH 7.5 at 4°C; 2.5 ml fractions were collected. The prolyl 3-hydroxylase activity (O, right ordinate) was assayed in 1.0 ml of the fraction and prolyl 4-hydroxylase activity (●, left ordinate) in 0.05 ml.

---

**Fig. 4. Separation of prolyl 4-hydroxylase tetramers and monomer-size protein by gel filtration from one control (a) and one injured (b) rat liver**

Samples (1.5 ml) of the 15000g supernatants of homogenates from the control and injured livers (9 ml of solution/g of liver) were used for gel filtration as described in Fig. 3. Immunoreactive prolyl 4-hydroxylase protein (●) was assayed in 0.2 ml of the fraction and the results are expressed as pg/ml of fraction.

The relationship between prolyl 4-hydroxylase activity and the amount of tetramers was studied further by examining the inhibition of the enzyme activity by various amounts of antiserum in the
gel-filtration fractions containing the enzyme tetramers. In this experiment, the enzyme activity in the sample from the injured liver was 3.3 times that in the control sample, and the amount of antiserum required for a 50% inhibition increased about 3.1-fold, indicating that the amount of antigen had increased to about the same extent as the enzyme activity (Fig. 5).

**Effect of hepatic injury on prolyl 4-hydroxylase protein in the serum**

The concentration of serum immunoreactive prolyl 4-hydroxylase was studied 48 h after induction of the liver injury with three different doses of dimethylnitrosamine (Fig. 6). A marked increase was found with 5 and 10 μl of dimethylnitrosamine/100 g body wt., the magnitude of the increase being clearly dependent on the dose.

Additional experiments indicated that serum immunoreactive prolyl 4-hydroxylase also increases in liver injury induced by carbon tetrachloride. When this compound was given in a dose of 100 μl/100 g body wt., the concentration of serum immunoreactive prolyl 4-hydroxylase in six rats was 1.9 ± 0.2 (s.d.) μg/ml, compared with 1.1 ± 0.2 (s.d.) μg/ml in six controls (P < 0.001; Student's t test).

**Discussion**

It has previously been reported that in hepatic injury prolyl 4-hydroxylase and lysyl hydroxylase activities increase earlier and to a greater extent than total prolyl 4-hydroxylase protein or collagen glucosyltransferase or glucosyltransferase activities (Risteli & Kivirikko, 1974, 1976; Risteli et al., 1976). One purpose now was to ascertain whether the change in prolyl 3-hydroxylase activity resembles that in prolyl 4-hydroxylase activity. The results indicated that it was possible to assay prolyl 3-hydroxylase activity reliably in liver samples and that it was identifiable as a separate prolyl hydroxylase, as reported for rat kidney cortex (Risteli et al., 1977; Tryggvason et al., 1977). The increase in prolyl 3-hydroxylase activity in liver injury was found to be very much smaller than that in prolyl 4-hydroxylase activity, and resembled the changes in total prolyl 4-hydroxylase protein. It has previously been noted that changes in collagen glucosyltransferase activities similarly resemble those in total prolyl 4-hydroxylase protein (Risteli & Kivirikko, 1976; Risteli et al., 1976).

The biological significance of the differential changes in the activities of individual intracellular

---

**Fig. 5. Inhibition of the activity of prolyl 4-hydroxylase tetramers from one control (○) and one injured (●) rat liver with increasing amounts of antiserum to rat prolyl 4-hydroxylase**

Samples (2.5 ml) of the 15,000 g supernatants of homogenates from the control and injured livers (5 ml of solution/g of liver) were used for gel filtration as described in Fig. 3. The positions of the tetramers were determined by the assay of prolyl 4-hydroxylase activity. Two fractions with the highest enzyme activity were pooled and inhibited with increasing amounts of antiserum. The enzyme activity in the sample from the injured liver was 3.3 times that in the control sample. The results are expressed in terms of the percentage inhibition observed. The broken lines show the amount of antiserum required for 50% inhibition in both samples. This was 3.1 times as much for the sample from the injured liver, indicating that the amount of antigen had increased to about the same extent as the enzyme activity.

---

**Fig. 6. Effect of acute liver injury induced by dimethylnitrosamine on serum prolyl 4-hydroxylase concentration**

The drug was administered intraperitoneally at doses of 1, 5 or 10 μl (diluted 1:100 or 1:25 with 0.15 M NaCl)/100 g body wt. 48 h before the blood samples were taken. Each treated group contained five rats and the control group six rats. The results are expressed as means ± S.D.
enzymes of collagen synthesis is not known. The additional collagen synthesized in early liver fibrosis is mainly type-III collagen (Kent et al., 1976; Rojkind & Martinez-Palomo, 1976), which has an approximately similar degree of prolyl 3-hydroxylation, lysyl hydroxylation and hydroxylysyl glycosylation as type-I collagen (see Martin et al., 1975; Epstein & Munderloh, 1975). Thus a change in the proportions of these two collagens synthesized should not in itself be accompanied by any alteration in enzyme activities. However, it is known that the extents of the post-translational modifications vary within the same type of collagen, and it has been pointed out previously (Risteli & Kivirikko, 1976) that the changes in lysyl hydroxylase and collagen glycosyltransferase activities in liver injury agree with those reported to take place in the extent of the corresponding post-translational modifications in dermal-scar and corneal-scar collagens (Cintron, 1974; Bailey et al., 1975; Shuttleworth et al., 1975).

Accordingly, if the increase in the rate of collagen biosynthesis is approximately similar to that in prolyl 4-hydroxylase activity, it is possible that the extent of prolyl 3-hydroxylation in the polypeptide chains might decrease, as a much smaller increase occurred in prolyl 3-hydroxylase activity in liver injury. Unfortunately no report of the 3-hydroxyproline content of collagen from fibrotic liver is available.

Although the change in total prolyl 4-hydroxylase protein was much smaller than in prolyl 4-hydroxylase activity, the increase in the amount of prolyl 4-hydroxylase tetramers was similar to that noted in the enzyme activity, the specific activity of the tetramers remaining unchanged. This finding agrees with previous reports indicating that under certain conditions alterations in prolyl 4-hydroxylase activity are associated with an unchanged specific activity of the tetramers (for references, see the introduction). It is nevertheless claimed that the increased prolyl 4-hydroxylase activity in the liver in experimental atherosclerosis may be attributed to an increase in the specific activity of the enzyme tetramers (Fuller et al., 1976). This conclusion was based on purification of the tetramers from livers of normal and atherosclerotic rabbits by affinity chromatography and on the finding that the $V_{max}$ of the tetramers from the latter source increased about 4-fold, with no change in the $K_m$. Unfortunately, the values reported concerned only one enzyme preparation from each source, and the degree of purification obtained was only about 600–700-fold. Since complete purification of prolyl 4-hydroxylase tetramers from an (NH$_4$)$_2$SO$_4$ fraction from newborn rats, which has a higher specific activity than a liver extract, is associated with an increase in specific activity of about 7800-fold (Risteli et al., 1976), it seems that the enzymes purified by Fuller et al. (1976) had lost most of their activity, and it is thus not known whether the inactivation was similar in the two enzyme preparations. It thus remains to be determined whether the case of liver in atherosclerosis differs from the others reported so far in being associated with increased specific activity of the enzyme tetramers.

The reason for the increase in the proportion of the enzyme tetramers in liver injury when expressed per total enzyme protein is not known, but this finding agrees with previous reports indicating that under conditions associated with a high rate of collagen biosynthesis a larger proportion of the enzyme protein is in the form of tetramers than is found at a low rate of collagen biosynthesis (Kao et al., 1975; Tuderman, 1976; Tuderman & Kivirikko, 1977). Recent data suggest that the monomer-size enzyme protein in the tissues represents, at least in part, precursors of the enzyme tetramers (Chichester et al., 1976; Tuderman et al., 1977), and therefore the increase in enzyme tetramers in liver injury may be due to a more efficient association of newly synthesized enzyme monomers to tetramers or an association of the pre-existing monomers.

Acute administration of dimethylnitrosamine is reported to induce selective hepatic injury characterized by cell necrosis and eventually leakage of intracellular enzymes into the serum (Chvapil et al., 1974). Accordingly it seems likely that the marked increase in serum immunoreactive prolyl 4-hydroxylase concentration found in the present study originated in the liver. Furthermore an increase in this protein was also found after acute carbon tetrachloride administration. But the data naturally do not exclude the possibility that some other tissue may have contributed to the increase in serum prolyl 4-hydroxylase. It has been reported previously that serum prolyl 4-hydroxylase activity increases in experimental liver fibrosis (Langness et al., 1975) and hepatocellular carcinoma (Keiser et al., 1972, 1975), and the present results suggest that this effect is indeed due to an increase in enzyme protein rather than a decrease in the amount of inhibitors (Stassen et al., 1974) present in the serum.

This work was supported in part by a grant from the Medical Research Council of the Academy of Finland. We gratefully acknowledge the expert technical assistance of Miss Helmi Konola and Mrs. Raija Pietilä.

References


1978
Takeuchi, T. & Prockop, D. J. (1969) Gastroenterology 56, 744–750