Intracellular Enzymes of Collagen Biosynthesis in Human Platelets

By Henrik Anttinen, Leena Tuderman, Aarne Oikarinen, and Kari I. Kivirikko

Activities of four intracellular enzymes of collagen biosynthesis—prolyl hydroxylase, lysyl hydroxylase, collagen galactosyltransferase, and collagen glucosyltransferase—were demonstrated in human platelets, and the presence of prolyl hydroxylase protein was further demonstrated by direct radioimmunoassay. The ratio of the specific activities of the four enzymes in the human platelet extract to those in human adult skin extract varied from about 0.1 to 1, the lowest relative activity being found with prolyl hydroxylase and the highest with collagen glucosyltransferase. Only a very small amount of prolyl hydroxylase protein, probably 1%, was in the form of the active enzyme tetramer. The collagen glucosyltransferase from human platelets readily glucosylated galactosylhydroxylysine in denatured collagen, but did not glucosylate native collagen. Also, native collagen did not act as an inhibitor of the glucosylation reaction. Therefore, platelet collagen glucosyltransferase cannot form either an enzyme–substrate complex or an enzyme–inhibitor complex with native collagen. The results thus argue against the theory which maintains that platelet collagen glucosyltransferase is involved in collagen–platelet adhesion.

Collagen-mediated platelet aggregation has been subjected to intensive investigation because of its postulated role in the formation of the hemostatic plug or atherosclerotic plaque. The process involves at least three distinct steps: the initial adhesion of platelets to collagen, the release reaction, and the subsequent platelet aggregation mediated by factors released from the platelets. Determinants at various structural levels of collagen have been reported to be responsible for this collagen platelet interaction. One determinant is to be found at the level of the collagen quaternary structure, which consists of the polymerized collagen fibrils. Also, the native triple-helical conformation of collagen is required for effective aggregation. However, denatured α1(I) chains of chick collagen, and even a cyanogen bromide peptide α1(I)-CB5 of chick skin collagen in high concentrations, can mediate the platelet release reaction and subsequent aggregation. The majority of the carbohydrate of the α1(I) chain is present in the α1(I)-CB5 peptide. Several reports have suggested the importance of collagen carbohydrates and also of certain polar amino groups in the recognition of collagen by the platelets. Thus, the primary structure of collagen also affects platelet aggregation.

Collagen galactosyltransferase and collagen glucosyltransferase have been demonstrated in platelets, and on this basis a theory has been put forward to explain the mechanism of collagen platelet adhesion, according to which...
membrane-bound collagen glucosyltransferase forms an enzyme-substrate complex with galactosylhydroxylysyl residues present in collagen. However, the methods used for the assays of collagen glycosyltransferases in the platelets have been criticized, and some properties of the platelet enzymes differ from those reported for collagen glycosyltransferases isolated from rat kidney cortex or chick embryos. There are numerous studies on the effect of collagen carbohydrate units on the collagen-platelet interaction, but the detailed substrate requirements of collagen glycosyltransferases in platelets are not known. It thus seemed to be of special interest to study if human platelet collagen glycosyltransferases can form a complex with intact triple-helical extracellular collagen, since collagen glycosyltransferases from chick embryos do not glycosylate native triple-helical collagen in vitro, and since the formation of collagen triple-helix prevents glycosylations in isolated chick embryo tendon and cartilage cells.

In the present work the presence of collagen galactosyltransferase and collagen glucosyltransferase activities in human platelets is re-investigated and the effect of the collagen conformation on the reaction catalyzed by human platelet collagen glucosyltransferase is studied in detail. In addition, the presence of two other intracellular enzymes of collagen biosynthesis in human platelets is reported, prolyl hydroxylase and lysyl hydroxylase.

**MATERIALS AND METHODS**

**Materials**

14C-Proline, 14C-lysine, UDP-14C-galactose, and UDP-14C-glucose were purchased from New England Nuclear Corporation, Boston, Mass. Unlabeled UDP-galactose and UDP-glucose were purchased from Sigma Chemical Company, St. Louis, Mo.

**Preparation of Platelet Extract**

Venous blood (100–200 ml) was drawn from healthy volunteers and anticoagulated with a solution consisting of 85 mM trisodium citrate, 65 mM citric acid, 2% glucose, and 6.6 mM EDTA (18 ml of solution/100 ml blood). All subsequent procedures were carried out at 0–4°C. Platelets were obtained by differential centrifugation, first twice for 15 min at 350 g to remove erythrocytes and leukocytes, and then for 10 min at 4300 g to obtain the platelet pellet. The platelets were washed twice with a solution consisting of 154 mM NaCl and 10 mM Tris HCI adjusted to pH 7.5 at 4°C. The purity of the platelet preparation was verified by light microscopy, and 3 erythrocytes and 3 leukocytes were found per 100,000 platelets. Washed platelets were homogenized in a Teflon and glass homogenizer with 60 strokes in a solution consisting of 200 mM NaCl, 100 mM glycine, 0.1%, Triton X-100, 50 μM dithiothreitol, and 10 mM Tris HCl buffer adjusted to pH 7.4 at 4°C. The homogenate was centrifuged for 30 min at 15,000 g, the supernatant being taken to constitute the platelet extract.

**Preparation of Collagen Substrates**

Denatured 14C-proline labeled or 14C-lysine labeled protocollagen substrates were prepared as described previously. Collagen substrate for the assays of collagen galactosyltransferase and collagen glucosyltransferase activities was prepared from calf skin and denatured by heating before the assay. Native citrate-soluble collagen was prepared from rat skin.

**Assays**

Prolyl and lysyl hydroxylase activities were assayed with 60,000 disintegrations per minute (dpm) of 14C-proline labeled or 110,000 dpm of 14C-lysine labeled protocollagen substrate.
COLLAGEN BIOSYNTHESIS

The amount of immunologically cross-reacting prolyl hydroxylase was measured by a specific radioimmunoassay, and the values were expressed as nanograms of pure human prolyl hydroxylase. Collagen galactosyltransferase and collagen glucosyltransferase activities were assayed as described previously, except that in most experiments 32 μM UDP-14C-galactose (specific activity 23 Ci/mole) for galactosyltransferase and 17 μM UDP-14C-glucose (specific activity 59 Ci/mole) for glucosyltransferase activity assays were used. Crude chick embryo ammonium sulphate enzyme was used as a standard for the assays of the four enzyme activities. The units of enzyme activity were expressed as reported previously.

The protein content of the platelet extract was assayed using bovine serum albumin as a standard.

Gel Filtration Study of Platelet Prolyl Hydroxylase

In order to study the composition of the total immunoreactive prolyl hydroxylase in the platelets, 1 ml of platelet extract was chromatographed on an agarose A 1.5-m column, and the fractions collected were assayed for immunoreactive prolyl hydroxylase content.

RESULTS

Intracellular Enzymes of Collagen Biosynthesis in Platelet Extract

Activities of prolyl hydroxylase, lysyl hydroxylase, collagen galactosyltransferase, and collagen glucosyltransferase were found in the platelet extract. Increasing amounts of enzyme reaction products were noted with the addition of increasing amounts of the platelet extract to the incubation mixtures (Fig. 1). The presence of prolyl hydroxylase protein was also demonstrated by direct radioimmunoassay (Fig. 1). The assays for prolyl hydroxylase and lysyl hydroxylase activities were entirely specific, as specific radiochemical procedures were used in the final assays.
of the products of these reactions. The assay procedure for the collagen glycosyltransferase activities was a modification of the original in that the paper electrophoresis step was omitted. In order to study the specificity of the modified method in the case of the platelet extract, the assays of collagen galactosyltransferase and collagen glucosyltransferase activity were carried out as in preparing the curves shown in Fig. 1, but the final products obtained were chromatographed in an amino acid analyzer using a program which clearly separated the hydroxylysine glycosides.

The results indicated that only about 50\% of the radioactivity chromatographed in the position of the galactosylhydroxylysine standard in the assay of collagen galactosyltransferase activity, whereas in the assay of collagen glucosyltransferase activity about 95\% was in the position of the glucosylgalactosylhydroxylysine standard (Fig. 2). Accordingly, the standard assay for collagen galactosyltransferase activity could not be considered specific enough to be used with platelet extract, and subsequent values were obtained by an assay method in which the final step was carried out using the amino acid analyzer. The assay for collagen glucosyltransferase activity, however, did show adequate specificity with the platelet extract.

The specific activities of the four enzymes and the concentration of prolyl hydroxylase protein in human platelet extract are summarized in Table 1. As the units of the four enzyme activities have different definitions, simple comparison of the activity levels in units of enzyme activity from the same source is not meaningful. Consequently, the activities of these enzymes in human skin extract, as determined in our laboratory, are also shown for comparison in Table 1. Collagen glucosyltransferase activity, when expressed per gram of extract protein, is similar in the platelet and skin extracts, while the other three enzyme activities are lower in the platelet extract, the lowest relative activity being found with prolyl hydroxylase.

The concentration of immunoreactive prolyl hydroxylase protein in the platelet extract is 0.18 mg/g platelet extract protein; this value is about one-tenth of that found in the skin extract (Table 1). When the units of prolyl hydroxylase activity are converted to milligrams of active enzyme protein, it may be calculated that only about 1% of the enzyme protein is in the active form. Prolyl hydroxylase protein has previously been shown to be present in tissues partly...
Table 1. Specific Activities or Concentrations of Intracellular Enzymes of Collagen Biosynthesis in Human Platelet and Skin Extracts From Adult Subjects

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Specific Activity or Concentration per Gram Extract Protein</th>
<th>Platelet Extract</th>
<th>Skin Extract*</th>
<th>Platelet/Skin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prolyl hydroxylase (U/g)†</td>
<td></td>
<td>0.1</td>
<td>1.3 (24)</td>
<td>0.1</td>
</tr>
<tr>
<td>Lysyl hydroxylase (U/g)†</td>
<td></td>
<td>12.0</td>
<td>60.0 (22)</td>
<td>0.2</td>
</tr>
<tr>
<td>Collagen galactosyltransferase (U/g)§</td>
<td></td>
<td>0.8</td>
<td>1.6 (28)</td>
<td>0.5</td>
</tr>
<tr>
<td>Collagen glucosyltransferase (U/g)‖</td>
<td></td>
<td>0.5</td>
<td>0.5 (28)</td>
<td>1.0</td>
</tr>
<tr>
<td>Immunoreactive prolyl hydroxylase (mg/g)</td>
<td></td>
<td>0.18</td>
<td>1.4 (24)</td>
<td>0.1</td>
</tr>
</tbody>
</table>

*Reference numbers are cited in parentheses.
†Prolyl hydroxylase unit of activity: the amount of enzyme synthesizing 1 μmole of hydroxyproline in 1 hr at 37°C using polypeptides (Pro-Pro-Gly), (n = 5, 10, 20) prepared by a solid-state method as substrates.18
‡Lysyl hydroxylase unit of activity: the amount of enzyme activity present in 1 mg of ammonium sulphate fraction (17%-45% saturation) obtained from the 15,000 g supernatant of chick embryo homogenate.22
§Collagen galactosyltransferase unit of activity: the amount of enzyme required to synthesize an amount of radioactive galactosylhydroxylysine in dpm corresponding to 1 μmole in 1 hr at 37°C using a saturating concentration of denatured purified citrate-soluble collagen as substrate.13
‖Collagen glucosyltransferase unit of activity: the amount of enzyme required to synthesize an amount of radioactive glucosylgalactosylhydroxylysine in dpm corresponding to 1 μmole in 1 hr at 37°C using a saturating concentration of denatured purified citrate-soluble collagen as substrate.12

In the form of the active enzyme tetramer and partly in the form of inactive enzyme protein corresponding in molecular weight to subunit monomers of the enzyme.242930 The form of prolyl hydroxylase protein in the platelet extract was studied by gel filtration, and the results indicated that essentially all the platelet prolyl hydroxylase protein is in a form corresponding in molecular weight to enzyme monomers (Fig. 3). This finding is in agreement with the result that only about 11% of the enzyme protein is active, as stated above.

Effect of Substrate Triple-Helical Conformation on the Platelet Collagen Glucosyltransferase-catalyzed Reaction

In order to study the effect of the collagen conformation on the reaction with platelet collagen glucosyltransferase, native and heat-denatured citrate-soluble collagen were compared as substrates for the enzyme. Since the denaturation temperature of citrate-soluble collagen at neutral pH is only about 38°C, the experiments were carried out at 30°C to ensure that the native collagen re-

![Fig. 3. Gel filtration of platelet prolyl hydroxylase. A 1-ml aliquot of platelet extract was chromatographed in an 8% agarose column, and fractions of 3 ml were assayed for immunoreactive prolyl hydroxylase protein. The first arrow indicates the elution position of the prolyl hydroxylase tetramer, and the second that of the prolyl hydroxylase monomer.](image-url)
mained entirely in its triple-helical conformation. In these experiments only the denatured collagen was found to act as a substrate for the glucose transfer reaction (Fig. 4).

Although collagen glucosyltransferase did not catalyze the reaction with native collagen as a substrate, the possibility still remained that triple-helical collagen could act as an inhibitor for the enzyme and that the enzyme would become bound to native collagen, forming an enzyme–inhibitor complex. Therefore, an experiment was performed in which the concentration of heat-denatured collagen was kept constant and various amounts of native collagen were added to the assay mixture. The denatured collagen concentration, chosen from the linear part of the product formation versus substrate concentration curve, was 0.2 g/liter (Table 2). Even a concentration of 1.2 g/liter of native collagen (a sixfold excess) did not inhibit glucose transfer to denatured collagen, indicating that native collagen is not an inhibitor of this reaction (Table 2).

**DISCUSSION**

The presence of four enzymes involved in the intracellular posttranslational modifications of collagen biosynthesis was demonstrated in human platelets. All these enzymes have been shown to be located within the cisternae of the endoplasmic reticulum of cells synthesizing collagen, and the enzymes may be present in this compartment as a multienzyme complex. Previously the presence of only two of these enzymes, the collagen glycosyltransferases, had been demonstrated in human platelets. However, due to their similar location in the collagen-synthesizing cells, it is not surprising that all four enzymes were present in the platelets.

The ratios of the specific activities of the four enzymes in human platelet extract to those in human skin extract vary from about 0.1 to 1, the lowest rela-

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**Table 2. Effect of Native Triple-Helical Collagen on Platelet Collagen Glucosyltransferase Activity**

| Denatured Citrate-soluble Collagen Substrate (g/liter) | Native Citrate-soluble Collagen (g/liter) | Enzyme Activity (dpm) | (% |)
<table>
<thead>
<tr>
<th></th>
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<tbody>
<tr>
<td>0.2</td>
<td>0</td>
<td>966</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>0.2</td>
<td>0.3</td>
<td>923</td>
<td>96</td>
<td></td>
</tr>
<tr>
<td>0.2</td>
<td>1.2</td>
<td>896</td>
<td>93</td>
<td></td>
</tr>
</tbody>
</table>
Collagen biosynthesis 35

Tive activity being found with prolyl hydroxylase and the highest with collagen


glicosyltransferase. Such variation in these enzyme activities is not surprising,


as considerable changes are known to take place with age and with a number of


conditions affecting collagen metabolism.30 For instance, lysyl hydroxylase ac-


tivity in human fetal skin is about 40-fold compared with that in human adult


skin,22 whereas collagen galactosyltransferase activity in human fetal skin is


only about 4 times that in human adult skin.28


Prolyl hydroxylase protein is found in tissues in two forms. The active en-


zyme is a tetramer having a molecular weight of about 240,000, but all tissues


contain in addition large amounts of a protein the molecular weight of which


corresponds to subunit monomers of the enzyme.24,29,30 The ratio between these


two forms of the enzyme protein varies between different tissues and as a func-


tion of age.24,29,30 For instance, about 15%-20% of the prolyl hydroxylase pro-


tein in human fetal skin is in the active tetramer form, whereas in human adult


skin only about 2%-4% is in this form.24 The present results indicate that only


about 1% of the prolyl hydroxylase protein is active in platelets, on the basis of


a comparison between enzyme activity and the concentration of the immuno-


reactive enzyme protein. Furthermore, gel filtration studies indicate that essen-


tially all the enzyme protein is in a form corresponding in molecular weight to


the enzyme monomers, but the presence of about 1% of enzyme tetramers


would probably not have been detected.


It is difficult to speculate on the reason for the presence of this enzyme pat-


tern in platelets. Platelets are capable of synthesizing small amounts of protein,


especially a contractile protein,1 but it is not known if they have an apparatus


for collagen biosynthesis. No hydroxyproline or hydroxylysine has been de-


tected in plasma membranes of human platelets, suggesting that they do not


contain collagen-like molecules.5


Effective collagen-mediated platelet aggregation requires the native triple-


helical structure of collagen,1,2 and vascular endothelial collagen obviously


exists in an intact triple-helical form. The results of this study indicate that


native triple-helical collagen cannot act either as a substrate or an inhibitor for


collagen glucosyltransferase in platelets. Accordingly, this enzyme cannot form


an enzyme-substrate complex or an enzyme-inhibitor complex with native


collagen, thus arguing against the theory which assigns a role to collagen


glicosyltransferase in collagen-platelet adhesion.5,7 It has recently been demon-


strated that none of these enzymes from chick embryos can catalyze the reac-


tion with native triple-helical collagen as a substrate,30 and it thus seems likely


that none can be involved in collagen-platelet adhesion either.


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