PROLYL HYDROXYLASE IN PLATELETS

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1. Introduction

Prolyl hydroxylase, a key enzyme of collagen biosynthesis, catalyses the hydroxylation of peptide-bound proline to hydroxy proline in a polypeptide precursor of collagen [1]. It is consistently found in connective tissue cells and in smaller amounts in certain non-fibroblastic cells, e.g., liver parenchyma, epithelium, melanoma, pituitary, kidney and neuroblastoma, several of which also synthesize collagen polypeptide [2–6]. Certain plant cell wall proteins contain hydroxyproline, and prolyl hydroxylase has been found in carrot discs [7].

Human blood serum contains small amounts of the enzyme [8,9] but little is known about its occurrence in the formed elements of the blood. Green and Goldberg found significant amounts in certain cultured lymphoma lines, which also contained traces of collagen polypeptide [2,3].

In view of the marked response of prolyl hydroxylase to vitamin C deficiency in cultured 3T6 fibroblasts [10], an approach to the detection of the early stages of functional vitamin C deficiency in animals and humans prompted an examination of blood-borne cells for this enzyme.

2. Materials and methods

2.1. *Preparation of enzyme extracts from blood cells and liver tissue*

About 30–60 ml blood from adult rats or from a human donor were anticoagulated with about 0.1 vol. 0.55 M sodium citrate, pH 7.4 and were layered in 3 ml aliquots over an equal volume of a blood separation mixture, density 1075, containing Triosil 440 (metrizoate radiographic contrast media from Nyegaard and Co., Oslo) and methyl cellulose [11]. After standing for 0.5–1.0 h, the upper layers containing the leucocytes and platelets were removed by suction and were centrifuged at 330 × *g* for 10 min; this sedimented virtually all the leucocytes and erythrocytes, leaving the platelets in suspension. The leucocyte-rich pellet was suspended in 0.87% ammonium chloride for 10 min to lyse the contaminating erythrocytes [12] and washed with buffered saline. Microscopic examination and differential counting in a Coulter Counter, model Z_{BI}, confirmed that platelets were virtually absent from the final leucocyte preparation and that leucocytes were virtually absent from the platelet-rich plasma. The platelets were collected by centrifugation of the platelet-rich plasma at 2400 × *g* for 20 min and washed with buffered saline. Erythrocytes were obtained from the lower layers of the blood separation mixtures, and washed several times with buffered saline.

Extracts for prolyl hydroxylase assay were prepared by gentle homogenisation, in a Potter-Elvehjem homogeniser, of the various blood cell fractions in a solution, pH 7.4, containing 0.2% Nonidet P-40 (a nonionic surfactant), 0.1 mM dithiothreitol, and 0.05 M Tris–chloride, followed by centrifugation at 2400 × *g* for 20 min to remove the insoluble residue. The leucocytes were homogenised in 0.5–1.0 ml of this buffer, yielding 2–5 mg soluble protein per ml; the platelets were homogenised in 1.0–2.0 ml, yielding 8–20 mg soluble protein per ml. The erythrocytes were homogenised in 9 vols of buffer yielding about 50 mg soluble protein per ml; samples of pooled rat liver were homogenised in 9 vols of buffer per gram wet weight, yielding about 20 mg soluble protein per ml. Protein was measured by the Folin method [13].

2.2. *Assay of prolyl hydroxylase in extracts*

Prolyl hydroxylase activity was measured by the release of ^3H_2O from underhydroxylated chick embryo substrate [14]. Although the batch of substrate used apparently had rather a low specific activity in terms of hydroxylatable proline residues, it also yielded low blank values and was adequate for the purposes of this investigation. Use of a more sensitive substrate would, of course, increase the sensitivity of the assay and permit the use of smaller amounts of enzyme extract.

0.3–0.5 ml Enzyme extract was incubated at 35°C for 60 min in 1.0 ml of a solution, pH 7.4, containing substrate (3.0 mg protein, 56 000 dpm), 0.1 M Tris–chloride, 0.012 M L-ascorbic acid, 0.001 M ferrous ammonium sulphate, 0.002 M α-ketoglutaric acid and 1800 units Sigma grade C-100 catalase. The reaction was stopped with 0.05 ml 100% w/v trichloroacetic acid, ^3H_2O was distilled at about 60°C and was measured by scintillation counting at 34% efficiency. Blank values were obtained by incubation of substrate plus cofactors and buffer, but without enzyme.

3. Results

Of the fractions from rat blood, only the platelet extract consistently yielded significant prolyl hydroxylase activity (table 1). Its specific activity was about one fifth of that of rat liver extracts prepared under similar conditions; several further experiments verified this result. Erythrocytes yielded no measurable activity; variable results were obtained with the leucocyte fraction, mainly because of the difficulty of obtaining pure leucocytes in sufficient numbers to give a concentrated extract. Plasma was inactive, but this was probably due to the presence of citrate, which is known to be inhibitory [8].

Table 1
Comparison of prolyl hydroxylase activities in extracts of rat blood fractions and of rat liver

Enzyme extract	Prolyl hydroxylase activity (dpm/min/mg protein/h incubation)	
	Experiment 1	Experiment 2
Rat liver	162	71
Rat plasma	<0.15	–
Rat erythrocytes	–	<0.15
Rat platelets	29	18

Preparation of enzyme extracts and the conditions of assay are described under Materials and methods.

Table 2
Cofactor-dependence of platelet prolyl hydroxylase

Cofactor omission	Activity relative to control
None (all cofactors present)	100
Minus α-ketoglutaric acid	<2
Minus ascorbic acid	12
Minus ferrous iron	20

All of the prolyl hydroxylase in the platelet extracts was soluble in the extraction medium; none could be detected in the insoluble residue after washing. Activity increased linearly with time up to 60 min and was absolutely dependent on added α-ketoglutaric acid; ascorbic acid and ferrous iron were also stimulatory (table 2).

Human blood yielded a similar picture: the platelet fraction alone contained measurable enzyme, whose specific activity was similar to that of the rat platelet extract. The leucocyte fraction, which was easier to prepare in a pure and concentrated form than that from rat blood, had less than 5% of the specific activity of the platelet extract. Addition of human leucocyte extract to rat liver extract failed to inhibit the enzyme present in the liver extract.

4. Discussion

The failure of the human leucocyte extract to catalyse hydroxylation was not, apparently, an artifact due to destruction of substrate by collagenolytic activity [15] or to inhibition by free radical or peroxidegenerating reactions [16] because the rat liver enzyme worked normally in its presence. It seems improbable that contaminating connective tissue cell, or fragments of them, could have been the source of the enzyme in the platelet fraction: those platelet samples which appeared entirely homogeneous under the microscope had specific activities at least as high as preparations which were obviously slightly contaminated with other cell types. Stein et al. [8] noted

that more prolyl hydroxylase could be detected in serum than in plasma; this difference might be attributable to release of the enzyme from platelets during clotting.

Platelets have recently been shown to possess the two glycosylating enzymes which add galactose, then glucose, to the hydroxylysyl residues of collagen [17–19] – they may, therefore, contain several components of the machinery of collagen synthesis. The possibility that platelets could in fact be capable of collagen synthesis under certain conditions in vivo has obvious relevance to wound healing and the fibrotic reactions associated with arterial disease.

If any of the platelet prolyl hydroxylase were present on the surface membranes of the cells, it might also play a role in collagen-induced platelet aggregation, since normal collagen contains some potentially hydroxylatable prolyl residues [20,21]. It is unlikely that this could constitute a major interaction site however, because hydroxylation requires single collagen chains [22,23], whereas platelet interaction favours triple helical, fibrillar collagen [24–26].

From a purely practical viewpoint the presence of prolyl hydroxylase in platelets might be a useful tool to investigate the physiological control and pathological responses of this key enzyme, since platelets are a convenient biopsy tissue for studies on human subjects.

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