SPECIFIC LABELLING OF PLATELET MEMBRANE GLYCOPROTEINS

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1. Introduction

The glycoproteins present on the platelet membrane are considered to be of great importance to platelet activities [1,2].

Few pathological platelet membrane glycoprotein abnormalities are known and usually they result in disturbance of aggregation. The Glanzmann's thrombasthenia is characterized by a defective mechanism of platelet aggregation induced by ADP, collagen and thrombin [3]. Decrease or even complete absence of glycoproteins II and one of the components (glycoproteins IIIa and IIIb) which contribute to the band known as glycoprotein III in platelets of patients suffering from this disease, has been reported [4–6]. Another pathological abnormality of platelet membrane glycoprotein is the Bernard-Soulier syndrome, which is characterized by reduced platelet adhesion to subendothelium. There is evidence that these platelets are abnormal in the distribution of glycoprotein Ib and glycocalicin [7,8]. The exposed position of the membrane glycoproteins on the platelet surface puts them in a good position to act as acceptors for aggregating agents or other stimuli. The determination of the steric location and availability of these glycoproteins is, therefore, of major importance and interest. We report here the design and use of a few reagents and methods to identify glycoproteins on platelet membrane. These reagents might be of utmost importance in the study of changes and defects in glycoproteins of platelet membrane and other cell membranes.

2. Materials and methods

2.1. *Platelet preparation*

Washed platelets were prepared as follows: Fresh human blood was drawn into ACD anticoagulant (1.5:10) and centrifuged at room temperature at 50 × *g* for 10 min. The PRP (platelet-rich plasma) obtained was centrifuged at 150 × *g* for 10 min to yield a platelet pellet. This pellet was resuspended in modified Tyrode's buffer (pH 7.2).

2.2. *3,5-Diiodo-L-tyrosine hydrazide*

(fig.1a) 3,5-diiodo-L-tyrosine, 1.065 g (2.5 mmol) was suspended in 25 ml dry methanol and cooled to −5°C. $SOCl_2$ (1 ml) was added dropwise. The solution became clear and was left at room temperature for 24 h, evaporated to dryness and the methyl ester was purified by column chromatography on silica gel and eluted with $CHCl_3$ 70%, methanol 30%. The total yield was 70%. The product was >98% pure (according to nitrogen analysis, NMR and mass spectrometry). The methyl ester (1 mg) was dissolved in 100 μl DMF (dimethyl formamide) and exchanged with 0.5 mCi ^{125}I, using ICl as catalyst [9]. To the reaction mixture 100 μl saturated NaCl solution was added and the precipitated product was collected by centrifugation in a Beckman Microfuge.

2.3. *Preparation of the hydrazide*

The radioactive methyl ester (0.5 mg) was dissolved in 0.5 ml methanol, 50 μl hydrazine hydrate were added and the mixture heated to 65°C for 3 h. The methanol was evaporated, the excess of hydrazine removed in vacuo and the hydrazide was purified by silica-gel column chromatography (elution with 5% methanol in methylene chloride). The compound co-migrated with a non-radioactive sample on thin-layer chromatography and, accordingly, was >95% pure.

2.4. *Diiodo-p-hydroxyphenyl acyl azide*

(a) Diiodo-*p*-hydroxyphenyl acetic acid: 5.84 g (38.4 mmol) of the *p*-hydroxyphenyl acetic acid

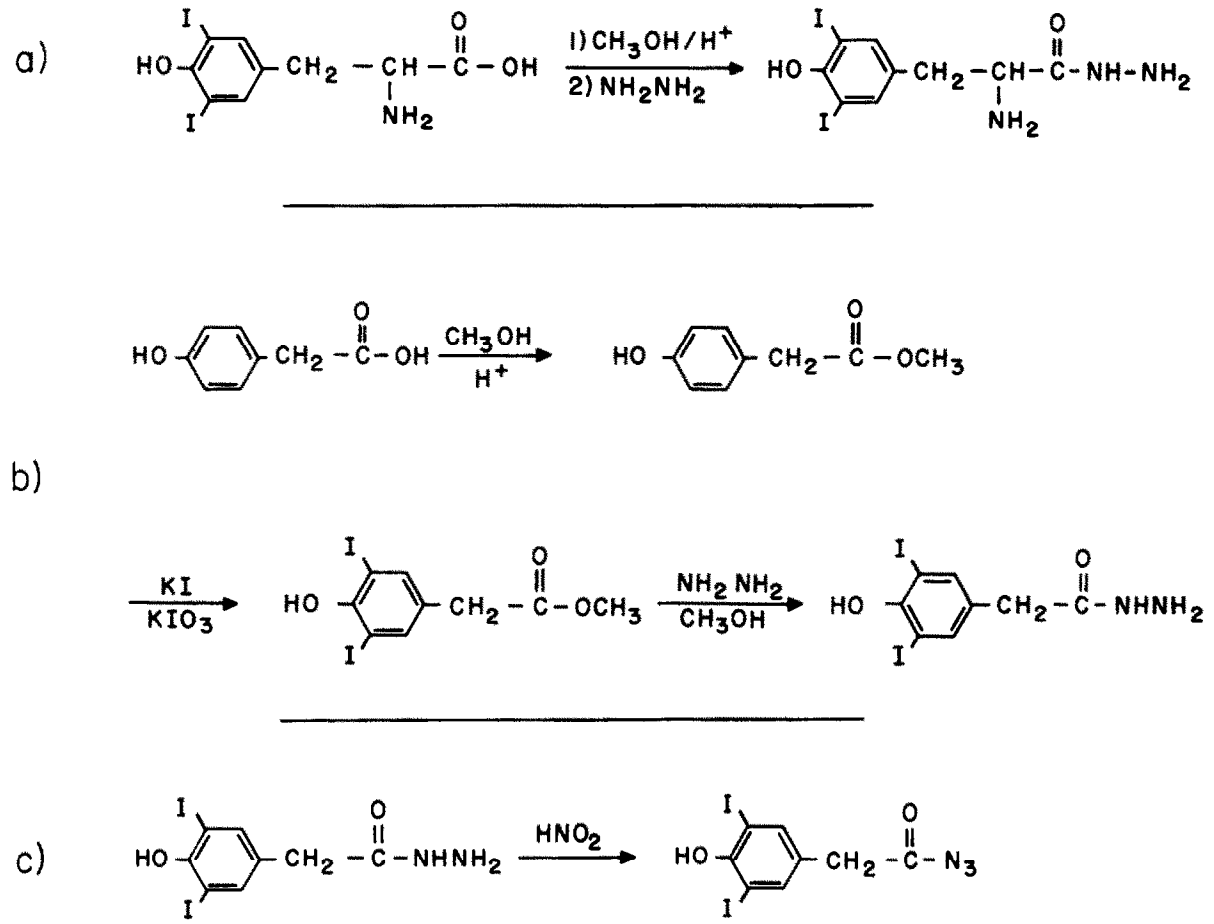

Fig.1. Synthesis of labelling reagents: (a) preparation of 3,5-diiodo-L-tyrosine hydrazide; (b) preparation of diiodo-*p*-hydroxyphenyl acyl hydrazide; (c) preparation of diiodo-*p*-hydroxyphenyl acyl azide.

were dissolved in 840 ml HCl 1 N and cooled to 3°C. A solution of 8.5 g KI + 5.5 g KIO_3 in 150 ml water was added dropwise. The brown precipitate obtained was filtered off, dissolved in 400 ml sodium bicarbonate (pH 8) and precipitated by acidification to pH 1 with concentrated HCl.

(b) Diiodo-hydroxyphenyl acetic acid methyl ester: 6.5 g diiodo acid were dissolved in 50 ml dry methanol containing 2 ml thionyl chloride, and left overnight at room temperature. After evaporation and three washings with methanol, the pale yellow product was washed with water and dried.

(c) Diiodo-*p*-hydroxyphenyl acyl hydrazide was prepared as described for 3,5-diiodo-L-tyrosine hydrazide (fig.1b).

(d) Diiodo-*p*-hydroxyphenyl acyl azide: prepared and used in situ as above (fig.1c).

2.5. *Labelling procedure with the hydrazide derivative*

The platelet suspension was treated with sodium periodate at 0.1–20 mM for 10 min at 10°C and then reacted with the hydrazide derivative at 5 mM and incubated at 5°C for 15 min. Sodium cyanoborohydride was added to 5 mM final conc. and the platelet suspension was incubated for another 10 min at room temperature.

2.6. *Labelling with NaB^3H_4 (sodium borohydride)*

Platelet suspension was treated with various concentrations of sodium periodate (0.2–20 mM) for 10 min at 10°C. After centrifuging and removing excess of the sodium periodate, the platelet suspension in saline containing 1 mM EDTA was treated with NaB^3H_4 (7 Ci/mmol) at 3×10^{-5} M final conc. for 15 min at room temperature. Control experiments

were done by reducing platelets with unlabelled $NaBH_4$ before the periodate oxidation or by omitting the periodate oxidation.

2.7. *Labelling with diiodo-p-hydroxyphenyl acyl azide*

Diiodo-*p*-hydroxyphenyl acetyl hydrazide, 2 mg/100 μl (50 mM) in 0.1 N HCl, were cooled to 0°C and treated with 0.1 M (final conc.) of sodium nitrite for 15 min. The solution was neutralized to pH 7.4 with sodium bicarbonate. This mixture (10 μl) was added to 750 μl platelet suspension in modified Tyrode's solution and the incubation was continued for 15 min at 10°C. The platelets were centrifuged in a Beckman Microfuge and the pellet was washed twice with saline containing 1 mM EDTA.

Exchange of unlabelled iodine with ^{125}I was carried out, using ICl as a catalyst [9].

SDS–polyacrylamide gel electrophoresis was done as in [10]. All gels were under reduced conditions.

NaB^3H_4 (7 Ci/mmol) and ^{125}I as KI carrier-free were purchased from Radiochemical Centre, Amersham. 3,4-Diiodo-L-tyrosine and *p*-hydroxyphenyl acetic acid were purchased from Sigma. Silica gel (kieselgel 60, 70–230 mesh) was purchased from Merck. Fluorography was done as in [11].

3. Results

3.1. *Synthesis of labelling reagents*

The various synthetic routes are described in fig.1. It is clear that the various steps involve fairly simple organic chemistry methods which produce very useful reagents.

3.2. *Periodate oxidation*

This method, developed in [17], is based on the difference in reactivity of various vicinal hydroxyl groups. The two hydroxyls on the sialic acid side chain are very sensitive to such oxidation and, therefore, the formation of aldehyde can be achieved in a very selective way. This aldehyde can be reduced, e.g., with NaB^3H_4, to produce the radioactive alcohol derivative of the sialic acid and the glycoprotein. It can also be reacted with the hydrazide derivative to form the labile Schiff base, which is stabilized by mild reduction with sodium cyanoborohydride. In both cases the end product is a modified glycoprotein which is labelled either by tritium or by iodine.

3.3. *Labelling of membrane glycoproteins with acyl-hydrazide derivatives*

3,5-Diiodo-L-tyrosine hydrazide proved to be a very convenient labelling compound and its synthesis was quite easy. When this reagent was reacted with whole platelets pretreated with 1 mM sodium periodate, a clear and specific labelling was obtained of a polypeptide having a M_r 150 000, probably glycoprotein I. When the periodate-treated platelets were reduced with NaB^3H_4, a completely different labelling pattern was obtained: mainly two polypeptides having M_r ~100 000. These might be glycoprotein IIIa and IIIb (fig.2).

4. Discussion

The labelling of platelet membrane glycoprotein described above is based on the same principle as the PAS staining, namely, the oxidation of vicinal hydroxyls with periodate, followed by a Schiff base formation. Use of the radioactive reagents increases

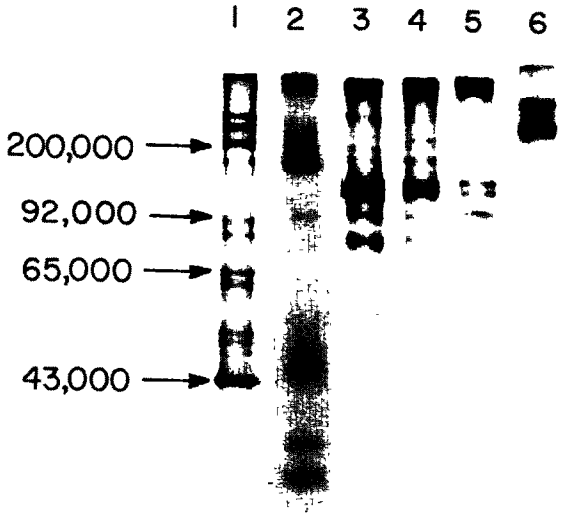

Fig.2. SDS–polyacrylamide electrophoresis and autoradiograms of labelled platelets: (1) Coomassie Blue staining of washed human platelets; (2) autoradiogram of whole platelets labelled with 3,5-diiodo-L-tyrosine hydrazide; (3) autoradiogram of whole platelets labelled with NaB^3H_4 after 20 mM periodate treatment; (4) same as (3) but the platelets were reduced with $NaBH_4$ (non-labelled) before the periodate treatment; (5) same as (3) but 0.1 mM periodate; (6) autoradiogram of whole platelets labelled with diiodo-*p*-hydroxyphenyl acyl azide.

the sensitivity over the conventional PAS methods. In addition, these methods enabled us to identify various membrane glycoproteins in the intact cell. The method of periodate oxidation, followed by NaB^3H_4 reduction, has been used in the past for the labelling of glycoproteins [12–14]. The various sugars exhibit different sensitivities towards periodate oxidation, sialic acid being one of the most sensitive, a fact that makes it possible to selectively label glycoproteins containing this acid.

Preparation of [^{125}I]tyrosine hydrazide, through the methyl ester, is very simple and results in a highly-polar compound, which will not easily penetrate the membrane lipid bilayer. The fact that the hydrazine is not directly attached to the aromatic ring only stabilizes the Schiff base formed and will thus increase the yield.

We used very low periodate concentrations (0.1–2 mM) for glycoprotein labelling, compared to those used in [14] or [12] (5–10 mM and 3–4 mM, respectively). Our results indicate that the periodate concentration had no effect on the labelling pattern. Two different labelling patterns were obtained when platelets were labelled with NaB^3H_4 or di[^{125}I]iodotyrosine hydrazide (fig.2). It is clear, therefore, that in both cases oxidation of sialic acid on glycoprotein I and glycoprotein III occurred. However, 3,5-diiodo-L-tyrosine hydrazide reacted mainly with the aldehyde groups formed on glycoprotein I, while NaB^3H_4 reduced mainly the aldehydes formed on glycoprotein III. It is possible that the differences in labelling are a result of the electrical and charge environment of the glycoproteins. While NaB^3H_4 reduction proceeds through a hydride ion $^3H^-$, i.e., a negative species, the interaction of the oxidized glycoprotein with 3,5-diiodo-L-tyrosine hydrazide is via the positively-charged hydrazide group. Therefore, it is possible that the immediate environment of glycoprotein III is less negative than that of glycoprotein I. This difference in electrical charge of the two glycoproteins might result from the presence of a high concentration of sialic acid on glycoprotein I [15].

Comparison of these labellings with that by diiodo-*p*-hydroxyphenylacyl azide, which will label proteins via ϵ-amino lysine groups, is shown in fig.2. No labelling of glycoproteins is obtained, but rather two proteins between M_r 200 000–260 000 are highly labelled.

We have shown that by proper design of the labelling reagent one can selectively label the glycoproteins on the platelet membrane. These labelling reagents are suitable for any glycoprotein and may, therefore, be used to label any other membrane glycoproteins in other cells.

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