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ISOLATION OF PLATELET PLASMA MEMBRANE PROTEINS BY AFFINITY CHROMATOGRAPHY

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Summary

A general method for the labelling and recovery of platelet membrane proteins is described. This method is based on the principle of labelling cell membrane proteins with reagents containing haptenic groups. The labelled proteins can then be isolated by affinity chromatography, using antibodies against the haptenic groups on the labelling reagents. Two compounds representing different groups of reagents were synthesized and used: 2,4-dinitrophenyl-β-alanine hydrazide, which is a labelling reagent for glycoproteins, and diazodiiodoarsanilic acid, which binds to externally exposed membrane proteins. 2,4-Dinitrophenyl-β-alanine hydrazide binds to aldehydes formed from oxidized sialic acid. Three main proteins were isolated using this reagent. Using diazodiiodoarsanilic acid, a protein having a molecular weight of 85 000 was labelled and later isolated in an affinity column with anti-arsanilic acid antibodies.

Introduction

The central role played by the protein components of membranes has been recognized, but progress in the isolation of these proteins, particularly integral membrane proteins, has been slow. The main reason for this slow progress is the particular molecular features of membrane proteins. Most of the known methods of isolation of membrane proteins are usually tedious and result in low yields. In these methods, the step of cell disruption and membrane solubilization preceded the isolation and hence the identification of the membrane proteins.

Abbreviation: SDS, sodium dodecyl sulfate.

Thus, many reports have appeared in the last few years concerning the solubilization of peripheral membrane proteins [1,2], as well as integral proteins [3,4]. These methods involve the solubilization of the membrane by non-ionic detergents [5] or ionic detergents [6], followed by gel filtration [7—9], ion-exchange chromatography [10—12] or affinity chromatography, usually with the use of lectins [13—16]. The method described here is based on the principle of the covalent labelling of membrane proteins in the intact cell, separation of the crude membrane, and then subsequent isolation of the labelled proteins by affinity chromatography, using antibodies against haptenic groups on the labelling reagents.

We used two labelling reagents on two cell systems : human blood platelets and erythrocytes. The first reagent is 2,4-dinitrophenyl-β-alanine hydrazide, which was prepared and used as a label for membrane glycoproteins. The second reagent prepared is diazo[^{125}I]diiodoarsanilic acid, which proved to be a non-penetrating reagent and is designed to label proteins containing tyrosine.

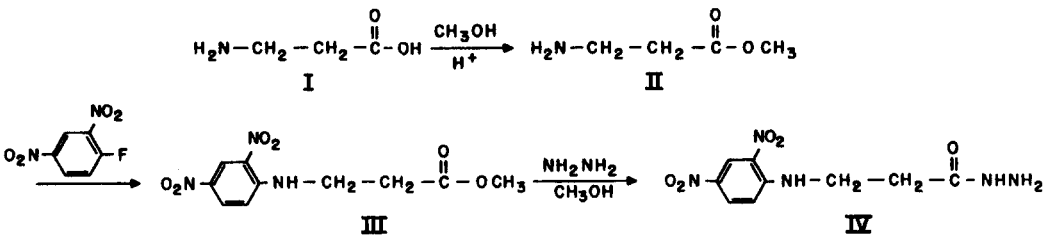
Materials and Methods

Preparation of labelling reagents

[^{125}I]Diiodoarsanilic acid. Diiodoarsanilic acid was prepared as follows: 2.17 g (0.01 mol) of arsanilic acid were dissolved in 20 ml of 1 M HCl and cooled to 4°C. A solution of 2.2 g KI and 1.4 g KIO_3 in 30 ml water was added dropwise. The grey precipitate obtained was filtered, dissolved in 4 ml $NaHCO_3$ (pH 8) and precipitated by acidification to pH 1 with conc. HCl. Purity was determined by thin-layer chromatography and iodine analysis.

[^{125}I]Diiodoarsanilic acid was prepared by exchange with diiodoarsanilic acid, according to the method of Helmkamp and Sears [17].

2,4-Dinitrophenyl-β-alanine hydrazide. 2,4-Dinitrophenyl-β-alanine hydrazide was prepared by reacting 2,4-dinitrofluorobenzene with β-alanine, as described by Rao and Sober [18]. The product was esterified by refluxing overnight with CH_3OH and HCl. The resulting methyl ester was boiled for 5 min in CH_3OH containing 10% N_2H_4 to yield a yellow precipitate of the hydrazide (Scheme I).



Scheme I. Preparation of 2,4-dinitrophenyl-β-alanine hydrazide. β-Alanine (I) is esterified with CH_3OH using acid catalysis to yield the β-alanine methyl ester (II). The ester is reacted with 2,4-dinitrofluorobenzene to yield 2,4-dinitrophenyl-β-alanine methyl ester (III) which is converted to the hydrazide (IV) by reaction with N_2H_4 in hot CH_3OH.

The product was identified according to NMR, mass spectra, positive 2,4,6-trinitrobenzenesulfonic acid, and nitrogen analysis (calculated, 26.0%; found, 25.8%). Melting-point = 125—127°C.

2,4-Dinitro[^{3}H]phenyl-β-alanine hydrazide. 2,4-Dinitro[^{3}H]phenyl-β-alanine hydrazide was prepared in a manner similar to that described for the non-radioactive material. 2 mg ($2 \cdot 10^{-2}$ mmol) of β-alanine methyl ester were incubated with 1 mCi of 2,4-dinitrofluoro[^{3}H]benzene in ethanol for 24 h at room temperature. The ethanol was evaporated under vacuum and the residue dissolved in ether and extracted with 0.1 M HCl. The non-aqueous layer was dried over Na_2SO_4 and the ether removed under vacuum. The crude product was purified by preparative thin-layer chromatography using the solvent system: 70% hexane/30% CH_2Cl_2. The pure methyl ester was converted into the hydrazide by heating at 60°C with 15 μl of $N_2H_4 \cdot H_2O$ and 50 μl of CH_3OH for 3 h. The hydrazide was purified by preparative thin-layer chromatography using the solvent system: 15% methanol/85% CH_2Cl_2. The purity of the compound was checked by co-chromatography with a non-radioactive sample.

Preparation of platelets

Platelets were obtaned as fresh platelet concentrates from the Central Blood Bank in Tel Aviv-Jaffa. They were centrifuged at $50 \times g$ for 15 min to precipitate residual red blood cells and then centrifuged again at $2000 \times g$ to yield the platelet pellet. This pellet was resuspended in modified Tyrode's buffer [19].

Labelling of cells

2,4-Dinitrophenyl-β-alanine hydrazide. Washed platelets were treated with 1 mM $NaIO_4$ at room temperature for 10 min. Excess IO_4^- was removed by centrifugation and 1 ml of cell suspension (containing approx. 5 mg protein) was reacted with 50 μl of a 10 mg/ml solution of the hydrazide derivative (final concentration $1.9 \cdot 10^{-3}$ M) for 10 min at 0°C. The free reagent was removed by centrifugation and the Schiff base formed was stabilized by reduction with $NaBH_3CN$ (20 μl of a 10 mg/ml solution for 10 min at room temperature).

Diazo[^{125}I]diiodoarsanilic acid. 200 μg of [^{125}I]diiodoarsanilic acid were dissoved in 150 μl of glacial acetic acid. To this solution, 10 μl of amyl nitrite were added at room temperature and the mixture was left at 10°C for 15 min. 5 ml of dry ether were added and the yellow diazonium salt precipitated. The supernatant was discarded and the dried pellet (kept in the cold and handled with care) was dissolved in 100 μl buffer (modified Tyrode's buffer or phosphate-buffered saline) and added to 500 μl suspension of $1 \cdot 10^8$ platelets. The mixture was incubated for 10 min at 4°C and the cells separated by centrifugation.

Anti-dinitrophenyl and anti-arsanilic acid antibodies were prepared by injection of 2,4-dinitrophenylhemocyanin and diiodoarsanilated hemocyanin, respectively, into rabbits, as described by Kabat [20]. The antibodies were purified by DEAE-cellulose ion-exchange chromatography [21].

Anti-dinitrophenyl and anti-arsanilic acid antibodies were coupled to Sepharose 4B, using the CNBr method as described by Wilchek and Gorecki [22]. The concentration of the antibodies was usually 10 mg/g wet Sepharose. The labelled platelets were lysed with dilute buffer (5 mM Tris-HCl, pH 7.2) and the suspension obtained was centrifuged at $40\,000 \times g$ for 30 min. The resulting

particulate fraction was dissolved in 2% Triton X-100 at room temperature for 30 min. Undissolved material was removed by centrifugation in a Beckman microfuge, and the supernatant was diluted with buffer to a Triton X-100 concentration of 0.2%. The particulate fraction (10 mg protein) solution was chromatographed on small columns (containing 3 ml of antibody/Sepharose suspension); after elution of 100 ml buffer, the labelled proteins were recovered by eluting the columns with 8 M urea or 10% HCO_2H. The urea or HCO_2H solution was dialyzed against H_2O for 3 days (4°C), lyophilized and the pattern of proteins determined by SDS-gel electrophoresis according to the method of Laemmli [23].

2,4-Dinitrofluoro[^{3}H]benzene (3 Ci/mmol) was purchased from Negev Radiochemicals (Israel). ^{125}I, as carrier-free NaI, was purchased from the Radiochemical Center, Amersham, U.K., β-alanine from Sigma, *p*-arsanilic acid from Fluka and $NaBH_3CN$ from Aldrich. The other chemicals used were of the highest purity available. Thin-layer chromatography was carried out on aluminium sheets silica gel 60 F_{254} pre-coated (Merck). Autoradiography was performed on vacuum-dried gels, using Kodak double-coated X-ray film. Fluorography was carried out according to the method of Bonner and Laskey [24].

Results

Labelling

2,4-Dinitrophenyl-β-alanine hydrazide. This labelling procedure is based on the well known Schiff reaction in which an aldehyde is reacted with an amine or a hydrazide. We generated the aldehydes by oxidation with IO_4^- of the vicinal hydroxyl groups of glycoprotein, sialic acid. The conditions used for the IO_4^- oxidation were mild, i.e., glycoproteins devoid of sialic acid will react only to a limited degree or not at all. This was done by using an IO_4^- concentration in the range of 1 mM [25]. Gel electrophoresis of whole platelets labelled with the tritiated reagent (Fig. 1) reveal labelling of at least seven proteins. Few of these proteins were not attracted by the affinity column and thus were not isolated. On the other hand, three other glycoproteins with molecular weights of 130 000, 100 000 and 65 000 were isolated.

Labelling with diazo[^{125}I]diiodoarsanilic acid. This reagent proved to be impermeant. When incubated with erythrocytes for 10 min at 4°C (final concentration of reagent $5 \cdot 10^{-4}$ M), only 8% of the radioactivity was associated with the globin (obtained from hemoglobin which served as an intracellular marker) while the rest (92%) of the label was covalently attached to the ghosts (data not shown). When human blood platelets were treated with this reagent under similar conditions, very specific labelling was obtained (Fig. 2). The main protein to be labelled has a molecular weight of 85 000—90 000.

Affinity chromatography

A Triton X-100 solution of the particulate fraction of labelled platelets with either of the labelling reagents was applied to the antibody affinity column. The pattern of proteins eluted from the anti-dinitrophenyl antibody Sepharose column of platelets labelled with 2,4-dinitrophenyl-β-alanine hydrazide is shown in Fig. 3. Three major proteins having molecular weights of 130 000,

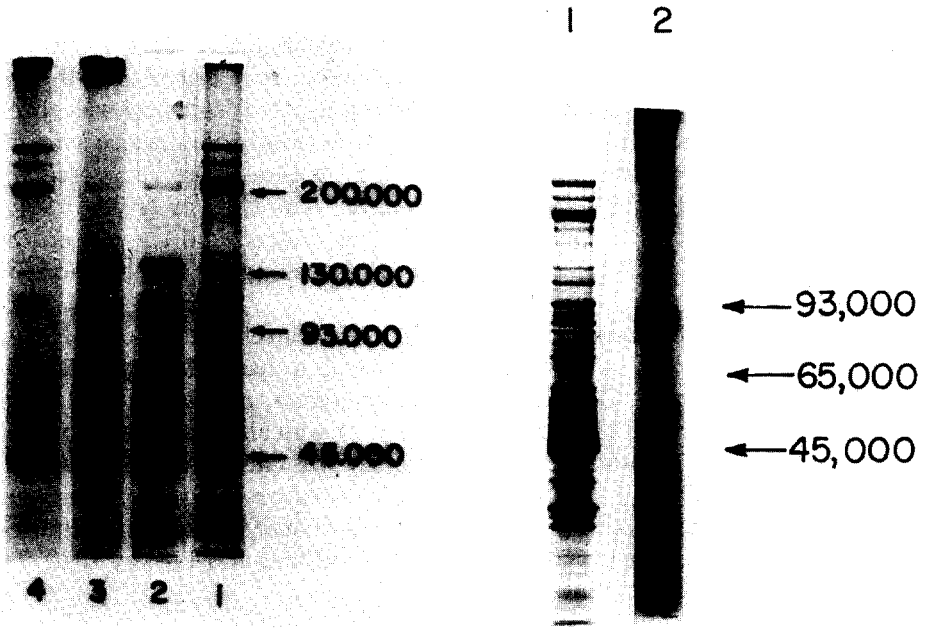


Fig. 1. SDS-polyacrylamide gel electrophoresis of platelets labelled with 2,4-dinitro[^{3}H]phenyl-β-alanine hydrazide. Gels were 5—15% gradients of acrylamide and samples were reduced with 10 mM dithiothreitol. Lane 1, Coomassie blue staining of whole platelets; lane 2, Coomassie blue staining of particulate fraction isolated from labelled platelets; lane 3, autoradiography of lane 2; lane 4, autoradiography of whole platelets labelled with 2,4-dinitro[^{3}H]phenyl-β-alanine hydrazide.

Fig. 2. SDS-polyacrylamide electrophoresis of whole platelets labelled with diazo[^{125}I]diiodoarsanilic acid (final concentration $5 \cdot 10^{-4}$ M), Lane 1, Coomassie blue staining of whole platelets; lane 2, autoradiography of labelled platelets.

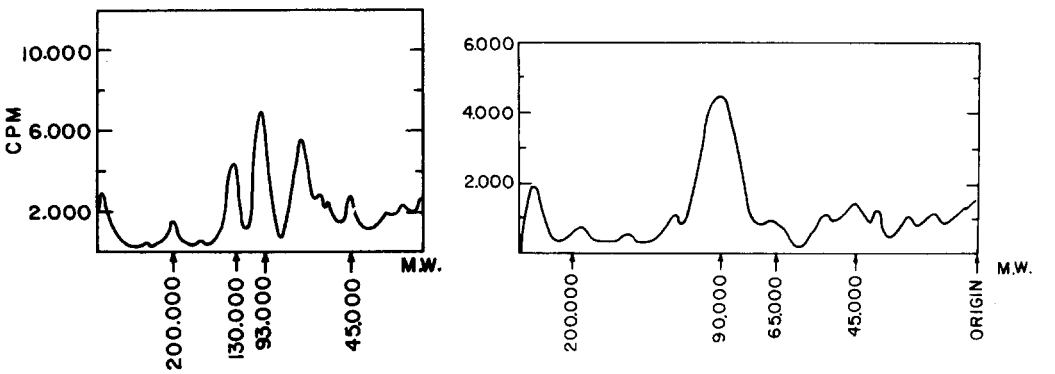


Fig. 3. Pattern of proteins eluted from chromatography of platelets labelled with 2,4-dinitro[^{3}H]phenyl-β-alanine hydrazide on anti-dintrophenyl antibody affinity column. (Gels were dried and sliced to a 1 mm slice width and counted.)

Fig. 4. Pattern of proteins eluted from chromatography of platelets labelled with diazo[^{125}I]diiodoarsanilic acid on antiarsanilic acid antibody affinity column. (Gels were sliced to a 1 mm slice width and counted.)

100 000 and 65 000 were isolated. According to Fig. 1, lane 4, more proteins in the intact platelets were labelled with this reagent, but could not be separated on these columns. Fig. 4 shows the profile of proteins eluted from an affinity column of anti-arsanilic acid antibodies coupled to Sepharose when the Triton X-100 solution of the particulate fraction of platelets labelled with diazoarsanilic acid was applied. In this case, the profile of isolated proteins is similar to the labelling pattern (Fig. 2, lane 2); the major protein obtained has a molecular weight of 85 000—90 000.

Discussion

This paper describes the use of chemical probes in the study and isolation of membrane proteins. The synthesis of these probes is fairly simple, the starting materials are commercially available chemicals, and radioactive, as well as non-radioactive, materials can be prepared quite easily. The probes chosen are based on compounds known to be good immunogens. As a result, these probes are recognised by the antibodies prepared and thus, a method of separation of the labelled antibodies was established. Two labelling reagents are shown here but, in principle, other reagents can be used provided that stable and active antibodies against the reagents can be prepared.

The advantage of this method is that the proteins are labelled in the intact cell and only afterwards is the integrity of the cells disrupted and the dissolved mixture chromatographed. Thus, not only were we able to label specifically glycoproteins or externally oriented proteins, but also a rapid separation of these proteins was achieved. One of the main difficulties in separating membrane proteins is the problem of solubilization of the proteins. Ionic and non-ionic detergents are widely used, but it is known that the antibody-antigen complex is sensitive to certain detergents at particular concentrations [26]. In this contribution, we overcame this problem by dissolving the platelet membranes in Triton X-100 at concentrations that maintain a stable antibody-antigen complex and thus enable efficient separation. Thus, we observed that dinitrophenyllysine or dinitrophenylbovine serum albumin are released from an anti-dinitrophenyl antibody Sepharose column when a buffer containing even 0.1% SDS was run through the column. On the other hand, the same antigen-antibody complex was stable at 2% Triton X-100.

We have no explanation for the fact that not all the glycoproteins labelled with dinitriphenyl-β-alanine hydrazide could be isolated by affinity chromatography. As this reagent is permeant, it might be that part of the proteins labelled is not of membrane origin. Another possibility is that part of the labelled proteins was not adsorbed to the affinity column.

The diazodiiodoarsanilic acid labelling pattern was very different from that obtained by George et al. [27,28] using a similar reagent, diazodiiodosulfanilic acid. In their elegant studies, George et al. [28] showed that diazodiiodosulfanilic acid preferentially labelled the three main glycoproteins on platelet membranes. On the other hand, the reagent which we used, diazodiiodoarsanilic acid, labelled mainly a protein having a molecular weight of approx. 85 000. A possible explanation might be the difference in the electrical charge of the two reagents which might influence the site of attachment by the reagent.

Another reason might be the different concentration used by us and by George et al. [28] in studies both of platelets and of the labelling reagent. Both reagents are quite impermeant as tested in human erythrocytes: diazodiiodosulfanilic acid showed 85% of the labelling in the membrane [29], while in our studies 92% of the labelling was located in the membrane and only 8% in the hemoglobulin.

Because of the relatively high polarity of diazodiiodoarsanilic acid, it is practically an impermeant reagent. Hubbard and Cohn [31] proposed criteria for the penetration of a label into the cell, based on the relative labelling of the membrane and the interior of the cell, and the relative amount of proteins in each fraction. Thus, if we assume that the membrane contributes 1% to the cell proteins and if 92% of the labelling is in the membrane (and 8% inside the cell), then the specific activity of the label is: $\frac{8}{92} \times \frac{1}{100} = 0.00087$. This value indicates a very low penetration of the label into the cell.

The advantage of using the arsanilic acid derivatives compared to those of sulfanilic acid lies in the very appealing possibility of isolating the labelled proteins by the use of affinity chromatography. Although antibodies against sulfanilic acid have been prepared previously [30], anti-arsanilic acid antibodies are much more commonly used and well characterized than those prepared against sulfanilic acid.

In the comparison of our results with other methods of platelet labelling and separation, we realized that the principal difference in our method is the introduction of the ligand for the affinity chromatography by an external probe, in contrast to the method used by Clemetson et al. [14] of using a wheat germ agglutination column. It is difficult to compare our glycoprotein labelling patterns with those of others because of the different reagent we used. Different labelling or detection methods give variations in results [32]; for example, McGregor et al. [33] reported a lack of staining of glycoproteins V and VI with the periodic acid Schiff technique. Thus, our reagent does not interact with all the membrane glycoproteins but only with a few; however, this interaction is sufficient to allow separation of the labelled proteins.

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