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AFFINITY CHROMATOGRAPHY PURIFICATION OF ERYTHROCYTE MEMBRANE PROTEINS AFTER SELECTIVE LABELING WITH TRINITROBENZENE SODIUM SULFONATE

GUIDO TARONE, MARIA PRAT and PAOLO M. COMOGLIO*

Department of Human Anatomy, Torino University, School of Medicine, 10126 Torino (Italy)

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SUMMARY

2,4,6-Trinitrobenzene sodium sulfonate may be used, under appropriate conditions, for specific surface labeling of erythrocyte plasma membranes. Trinitrophenylated ghost proteins are easily purified by reverse immunoadsorption using rabbit anti-dinitrophenyl antibodies covalently linked to Sepharose 4B. The advantages of a method that permits selective labeling of membrane molecules with a hapten and their purification by affinity chromatography are obvious. The possible applications of such a method in the investigation of the composition of plasma membranes are discussed.

INTRODUCTION

Several techniques have been used to study the composition of cell plasma membranes. Methods have been described for obtaining purified plasma membranes from cell homogenates[1–6] or for the selective solubilisation of surface membrane components by enzymatic digestion[7,8] of intact cells. Alternatively, cell surface molecules have been specifically labeled with reagents that poorly penetrate the cell[9–12].

Each method has its own advantages and disadvantages. Isolation of purified plasma membrane fractions, for example, does not allow an easy distinction between structural membrane molecules and cell-soluble components adsorbed on the membranes after cell disruption. Enzymatic digestion largely prevents such contamination but in almost all cases splits membrane molecules into components of lower molecular weight. Methods for the specific labeling of surface membrane proteins often give excellent results but are essentially analytical. In this paper, we describe a method for labeling cell surface molecules with trinitrophenyl groups. This method has the advantage of allowing easy separation of labeled plasma membrane molecules owing to the well-known immunochemical properties of trinitrophenyl derivatives. Erythrocytes were used as an experimental model since their soluble cytoplasmic proteins, mostly hemoglobin, are optically identifiable without difficulty. The ability

* Author to whom correspondence should be addressed.

of this method to recover hemoglobin-free molecules from erythrocyte ghosts was considered a criterion of its suitability for labeling and purifying membrane proteins.

MATERIALS AND METHODS

Cells

Human red blood cells from volunteer donors were used after five washings with Alsewer's solution, pH 6.0, to remove the buffy coat and serum proteins.

Reagents

Chemicals were purchased from the following sources: 2,4-dinitrobenzenesulfonic acid (sodium salt), 2,4,6-trinitrobenzenesulfonic acid (sodium salt) and cyanogen bromide from Eastman Kodak; Sepharose 4B from Pharmacia and sodium deoxycholate from Merck. Human serum albumin and bovine γ-globulin were obtained from Sigma. Dinitrobenzenesulfonate was twice recrystallized from ethanol and trinitrobenzenesulfonate from 1 M HCl. Dinitrophenyl$_{47}$–human serum albumin and dinitrophenyl$_{60}$–bovine γ-globulin were prepared according to the methods given in detail by Eisen *et al.*[13].

Preparation of antibodies and immunoadsorbent

New Zealand rabbits were by immunized by injections at 3-month intervals of 1 mg of dinitrophenyl$_{47}$–human serum albumin in Freund's complete adjuvant subdivided in several subcutaneous sites. Seven days after the last booster, rabbits were bled by cardiac puncture and sera containing approx. 2–3 mg of anti-dinitrophenyl antibodies/ml were harvested and stored frozen until used. Rabbit anti-dinitrophenyl antibodies were purified using an immunoadsorbent made with Sepharose 4B coupled to dinitrophenyl$_{60}$–bovine γ-globulin by cyanogen bromide technique[14,15]. 15 mg of antigen were coupled to each g of activated Sepharose. Purified antibodies were eluted by 10 mM dinitrophenylglycine buffered at pH 8.0, chromatographed on Dowex 1-X8 ion-exchange resin (Cl^- form; 20–50 mesh), dialysed against 0.1 M sodium bicarbonate, pH 9, and used to prepare a reverted immunoadsorbent. 25 mg of purified antibodies were reacted overnight at 4 °C with each g of activated Sepharose. Under these conditions, 80% of the antibodies were covalently linked to the resin.

Spectrophotometric analysis

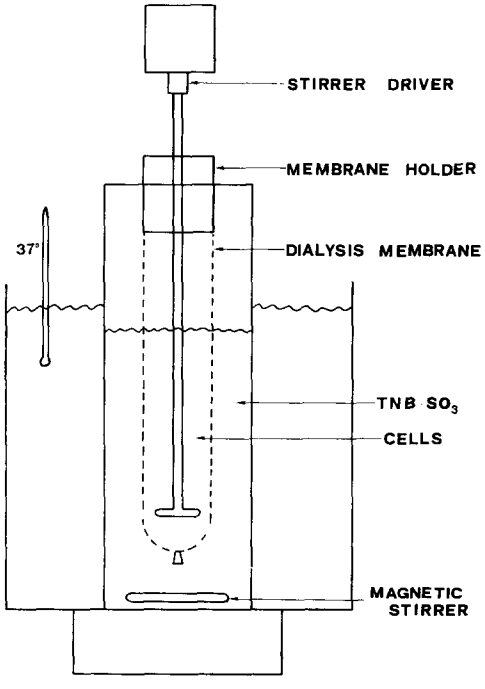
Trinitrophenyl groups were determined quantitatively by light absorption at 348 nm and protein concentrations at 278 nm. The amount of trinitrophenyl groups linked to soluble or solubilized ghost proteins was calculated from the ration between the absorbances at 348 nm and 278 nm, after correction for hemoglobin interference at 348 nm. Both hemoglobin and trinitrophenol absorb at 348 nm. To eliminate this possible source of interference to the absorbance of the sample at 348 nm, a value Y obtained in the following way was subtracted: $A_{550\ nm}$ of the sample $\times K = Y$, where $K = A_{348\ nm}/A_{550\ nm} = 2.4$.

The constant K has been calculated on the spectrum of trinitrophenyl unlabeled hemoglobin samples. The absorbance value at 550 nm has been selected for being far enough from the other spectral peaks to avoid any interference.

RESULTS

Labeling of membrane proteins

Several labeling experiments were performed by reacting human erythrocytes with trinitrobenzenesulfonate at various concentrations, temperatures and pH. The amount of trinitrophenyl groups coupled to surface and cytoplasmic-soluble erythrocyte proteins varied in each condition. Optimum selective surface labeling results were obtained when the reaction occurred at pH 7, at 37 °C, while the concentration of trinitrophenylsulfonate increased progressively from 0 to a prefixed value during incubation. To determine this concentration an apparatus was built (Fig. 1) in which red cells ($3 \cdot 10^8$ cells/ml) suspended in 0.15 M NaCl, 0.01 M phosphate buffer, pH 7.15, were separated by a dialysis membrane from a 15 mM trinitrobenzenesulfonate solution in phosphate-buffered saline. Both the cell suspension and the trinitrobenzenesulfonate solution were stirred and heated to 37 °C. The reaction was performed for 2 h and samples were taken every 10 min. The reaction was stopped by several washings in iced 750 mM glycine until centrifugation gave colorless supernatants. The absorbance of trinitrobenzenesulfonate and the pH of each first washing supernatant are shown in Fig. 2. Cell lysis in each sample was evaluated



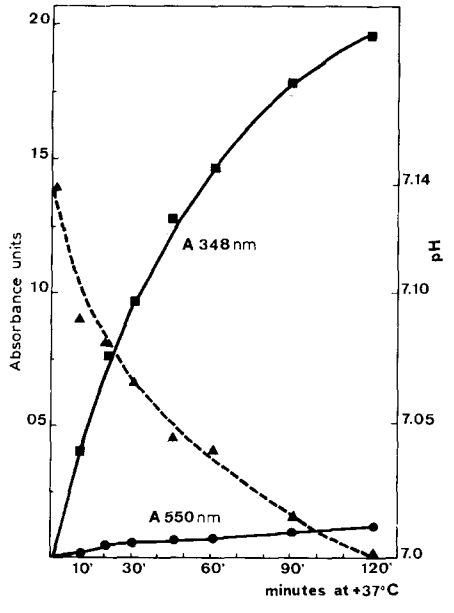


Fig. 1. Schematic diagram of the apparatus employed for the surface labeling of erythrocytes. Cells suspended in phosphate-buffered saline were separated by a dialysis membrane bag from the trinitrobenzenesulfonate (TNB-SO_3) solution. Both the suspension and the reagent solution were constantly subjected to gentle stirring.

Fig. 2. Free trinitrobenzenesulfonate concentrations and hemoglobin release into the dialysis bag during erythrocyte labeling at 37 °C. ■, absorbance at 348 nm; ●, absorbance at 550 nm; ▲, pH.

by measuring hemoglobin release, monitored by the absorbance of the first supernatant at 550 nm. As can be seen in Fig. 2 hemolysis which occurred under these labeling conditions was very low. Surface labeling was tested by agglutination with rabbit anti-trinitrophenyl antibodies; positive results were obtained after only 10 min of incubation. To quantitate trinitrophenyl groups linked to membrane or soluble erythrocyte proteins, cells were lysed by osmotic shock in distilled water and centrifuged once at 100000 $\times g$ for 1 h; supernatants were saved and ghosts, which were heavily contaminated with hemoglobin, were then solubilized in 3 vol. of 1% deoxycholate in 0.1 M barbital buffer, pH 8.3. After centrifugation at 100000 $\times g$ for 1 h, clear supernatants were obtained. Cytoplasmic fractions and solubilized ghosts were then extensively dialysed against 1% deoxycholate in the same buffer. Samples diluted 3:10 in Merckotest (Merck) were tested for trinitrophenyl groups. Labeling was expressed as the ratio between absorbance at 348 nm, subtracted for the value of hemoglobin, and 278 nm. The typical picture (Fig. 3) was of insignificant hemoglobin labeling during the first h of reaction, whereas ghost protein values increased exponentially for 2 h without saturation. Optimal differentiation between surface and cytoplasmic molecule labeling was obtained by stopping the reaction after 30 min, when the free concentration of the reagent inside the dialysis bag reached 4–5 mM.

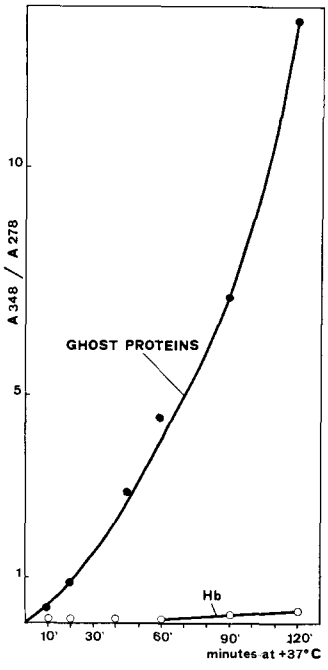


Fig. 3. Trinitrophenyl groups linked to ghost proteins and hemoglobin in erythrocytes labeled with trinitrobenzene sulfonate for different incubation time at 37 °C. Labeling of hemoglobin was insignificant for the first h.

At that time, ghost protein labeling was greater than 100-fold that of hemoglobin (see Table I). Differences of this extent made purification of trinitrophenyl-labeled proteins very easy. Hemoglobin prepared from the same number of lysed red blood

cells and labeled by trinitrobenzenesulfonate under identical conditions was used as the control. Its $A_{348\,nm}/A_{278\,nm}$ ratio was 0.38 (see Spectrophotometric analysis section).

TABLE I

Preparation labeled	*Fraction*	$\frac{A_{348\ nm\ (trinitrophenol)}}{A_{278\ nm}} \times 10^{-2}$
Red cells	Ghosts	200
Hemoglobin	Hemoglobin	3
	Hemoglobin	38

Purification of labeled proteins

$1 \cdot 10^{10}$ red blood cells, labeled for 30 min as described, were lysed and ghosts were separated by centrifugation. After solubilisation in 10 ml 1% deoxycholate in 0.1 M barbital buffer, pH 8.3, and extensive dialysis against phosphate-buffered saline, ghost proteins heavily contaminated with hemoglobin were passed through a 1 cm × 12 cm column of purified rabbit anti-dinitrophenyl antibodies linked to Sepharose. These anti-dinitrophenyl antibodies cross-react with trinitrophenylated antigens and were preferred to anti-trinitrophenyl antibodies, since the high affinity of rabbit antibodies for their specific nitrophenylated ligands prevents elution of the trinitrophenyl proteins except under very strong conditions, with the possible denaturation or release of antibodies covalently linked to Sepharose[18-22]. The elution pattern of our immunoadsorbent is reported in Fig. 4. After sample application, the column was eluted by phosphate-buffered saline at pH 7.25 and 0.5-ml fractions were collected.

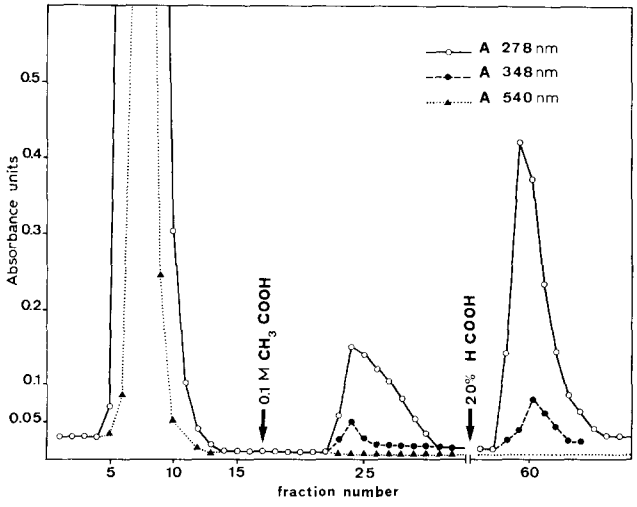


Fig. 4. Purification of trinitrophenyl-labeled ghost proteins on anti-dinitrophenyl–Sepharose column. After sample application, the column was washed with phosphate-buffered saline, pH 7.25, and eluted by 0.1 M acetic acid, followed by further elution with 20% formic acid (arrows). In each fraction, protein concentration was monitored by absorbance at 278 nm, trinitrophenyl groups at 348 nm and hemoglobin at 550 nm.

The emerging peak contained the hemoglobin adsorbed on ghosts after cell lysis. After extensive washing of the column with phosphate-buffered saline, trinitrophenyl proteins were recovered in pure form in a second peak eluted by 0.1 M acetic acid. This peak absorbed at 278 nm and 348 nm, but showed no absorption at 550 nm, indicating complete freedom from hemoglobin. The trinitrophenyl protein yield was in the range of 40%, as shown by further elution with 20% formic acid to strip the remaining trinitrophenyl proteins from the column. Yields may be doubled by elution with defolding agents, such as 8 M urea or 7 M guanidine, or by elution with dinitrophenyl haptens. Here, however, the immunoadsorbent loses its activity and cannot be reutilised. Trinitrophenyl proteins thus purified from erythrocyte membranes and run on sodium dodecyl sulfate acrylamide gels at pH 8.5 were separated in three different electrophoretic peaks. The same experiment was repeated with trinitrophenyl proteins purified from erythrocyte ghosts which were first solubilized by deoxycholate and then labeled under the same conditions. In this case the electrophoretic pattern was very complicated. At least twelve more bands could be seen clearly, one of which corresponded to trinitrophenylhemoglobin.

DISCUSSION

Affinity chromatography on Sepharose columns conjugated with antibodies has been shown to be a useful method for the purification of a variety of native proteins[14,16–20]. A method for the purification of peptides containing modified tyrosine residues using anti-dinitrophenyl antibodies conjugated to Sepharose has also been described[21]. Our present results suggest that affinity chromatography is also feasible for the purification of plasma membrane proteins. In this connection, it should be recalled that conventional physicochemical methods raise a number of difficulties, many of which would be avoided by the employment of specific antibodies. These antibodies, however, cannot readily be produced in sufficient quantities for the preparation of immunoadsorbents, though this problem can be got round by the artificial introduction of a given antigenic determinant into the membrane proteins. For this purpose, we used a trinitrophenyl compound, since the immunochemical properties of this group are well known, anti-trinitrophenyl or anti-dinitrophenyl antibodies can be produced in large quantities without undue difficulty and erythrocyte plasma membrane proteins can be labeled selectively with trinitrobenzenesulfonate without labeling the cytoplasmic hemoglobin[23]. Affinity chromatography was used to separate the labeled proteins and three peaks were obtained on sodium dodecyl sulfate acrylamide gels. It has been suggested[11,12] that only one, or at most no more than a few, proteins are to be found on the human erythrocyte plasma membrane surface, and no explanation can as yet be given for these three peaks, particularly since nothing was done to prevent proteolytic degradation during solubilisation of the ghosts. This point has not been pursued, since we were not concerned with erythrocyte membrane structural questions. It does, however, indicate that only a few proteins are labeled when the membrane is unimpaired and many more after its disorganisation.

Further work (to be published separately) has shown that trinitrobenzenesulfonate can be successfully employed on other cell types by slight variation of the specific labeling conditions. The advantages offered by a method that permits the

specific labeling of membrane molecules with a hapten and their subsequent purification *via* the immunochemical properties of the label are obvious. The position of labeled molecules can be determined in conditions that are physiological for the cell. They can also be obtained in a pure form by means of a single step, so that subsequent handlings required for conventional macromolecule separation are no longer necessary.

The only drawback of the method lies in the non-total impermeability of the membrane to the trinitrobenzenesulfonate. This, coupled with the fact that the reaction is relatively slow, means that some trinitrobenzenesulfonate molecules may penetrate (or be carried inside) the cell. Screening of other reagents might lead to the identification of one or more compounds, with an antigen activity equal to that of the nitrophenyl derivatives, that are easier to use than trinitrobenzenesulfonate and have the added advantage of being able to cross the membrane. At all events, it is clear from our results that suitable adjustment of reaction time and temperature and reagent concentration enables trinitrobenzenesulfonate to display a 100–300-fold superiority in binding trinitrophenyl groups to plasma membrane as opposed to cytoplasmic proteins. Differential labeling of these proportions means that no difficulty is encountered in purifying labeled from unlabeled molecules by immunoadsorption. As described here, the process is concerned with red cell membranes. Its adaptation to the study of the composition of other cell membranes should not be a difficult matter.

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