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A CATIONIC HYDROXYSUCCINIMIDE ESTER

A REAGENT FOR LABELING EXTERIOR MEMBRANE PROTEINS

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Summary

^{3}H-labeled *N,N,N*,trimethylamino-β-alanyl-*N*-hydroxysuccinimido ester ([^{3}H]TMAS), a new cationic membrane reagent, was synthesized. TMAS was shown to be impermeant through human erythrocyte membranes. Under mild physiological conditions TMAS reacted primarily with amino groups of the membrane proteins and lipids. The pattern of erythrocyte proteins labeled with [^{3}H]TMAS was examined by polyacrylamide gel electrophoresis under denaturing conditions. Externally oriented labeling of intact erythrocytes revealed a major radioactive protein with an apparent molecular weight of 90000. By labeling ghost preparations with [^{3}H]TMAS the radioactivity incorporated into all the major Coomassie Brilliant Blue bands resolved by gel electrophoresis.

The agreement between the results obtained with anionic and cationic amino reactive probes indicates that the ionic character of the reagent has a minor effect on the pattern of labeled exterior polypeptides observed in erythrocytes.

Introduction

Considerable effort has been invested in recent years in the elucidation of the structure of plasma membrane proteins. For this purpose a variety of enzymatic and chemical impermeant membrane probes have been used [1—15]. All these reagents are found to form addition products with reactive groups on the exterior surface of the erythrocyte membrane.

Most of the chemical reagents hitherto used are of an anionic character and may have a greater affinity towards cationic sites on the surface membranes. To

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Abbreviation: TMAS, *N,N,N*,trimethylamino-β-alanyl-*N*-hydroxysuccinimido ester.

complete the scope of membrane probes it is desirable to devise a cationic reagent that would be accessible to anionic membranal proteins. The present report describes the synthesis of *N,N,N*,trimethylamino-β-alanyl *N*-hydroxysuccinimido ester (TMAS) which meets the above requirements. TMAS was found to be impermeable into erythrocytes, and to react under mild physiological conditions with amino groups on the membrane surface.

This reagent was synthesized with the future aim of applying it in the labeling of nerve cells where cationic ligands are thought to play an important role in triggering the excitable membranes.

Materials and Methods

Preparation of TMAS

N,N,Dimethylamino-β-alanine was prepared by reacting β-alanine with an excess of formaldehyde in 90% formic acid. The mixture was heated on a steam bath for 8 h. Excess reagent was then removed by repetitive washing with water followed by flash evaporation.

The *N*-hydroxysuccinimidoester of dimethylamino-β-alanine was prepared by the method of Anderson et al. [16]. The succinimido ester was dissolved in ethanol and 30% excess of methyliodide was added, and the solution was then left overnight, at room temperature. Considerable amounts of the quaternary methiodide separated. The white solid product was washed with peroxide-free dry ethyl ether and kept below 0°C. The yield of TMAS based on β-alanine was 40—45%. Neutral equivalent [17], was determined to be 344 (calculated 355). Thin layer chromatography on silica gel (Merck thin layer chromatography plates) with *n*-butanol/acetic acid/water (1 : 1 : 1, v/v) revealed a single iodine reactive spot.

Synthesis of [^{3}H]TMAS

Generally ^{3}H-labeled *N,N*,dimethylamino-β-alanine (500 mCi/mmol) was prepared by catalytic exchange with tritium gas by the Israel Nuclear Research Center - Negev. [^{3}H]TMAS (80 mCi/mmol) was prepared from ^{3}H-labeled *N,N*,-dimethylamino-β-alanine as described above. Purity of the radioactive compound was examined by thin layer chromatography.

Preparation of human erythrocytes, erythrocytes ghosts and heme-free globin

Human erythrocytes were prepared from freshly drawn human blood in acid citrate dextrose. The cells were washed three times with 20 vols. of cold isotonic phosphate buffer, pH 7.4 (50 mM phosphate/84 mM Cl^-/176 mM Na^+). Hemoglobin-free ghosts were isolated from washed erythrocytes after hypotonic lysis [18]. Heme-free globin was prepared by acetone-HCl extraction [19].

Labeling of intact erythrocytes

Washed erythrocytes suspension (10%, v/v) in isotonic phosphate buffer, pH 8.0, (50 mM phosphate/175 mM Na^+/82 mM Cl^-/3.6 mM K^+) was incubated at 37°C for 1—2 h with 1—5 mM [^{3}H]TMAS (50—100 mCi/mmol). The cells were then washed thrice by centrifugation with 30 vols. of isotonic phosphate buf-

fer, pH 7.4, to remove excess reagent. The packed cells were lysed in 50 vols. of hypotonic 5 mM phosphate buffer, pH 7.4. Erythrocyte membranes were isolated and the hemoglobin was used to prepare heme-free globin. Membranes were solubilized in 1% mercaptoethanol containing 1% sodium dodecyl sulfate (SDS), and boiled for 5 min to avoid proteolysis.

Labeling of erythrocyte membranes and of lysed cells

Erythrocyte ghosts suspended in 50 vols. of hypotonic 5 mM phosphate buffer, pH 8.0, and washed erythrocytes lysed in 50 vols. of hypotonic 5 mM phosphate buffer, pH 8.0, were incubated with 1—5 mM [^{3}H]TMAS, at 37°C. The ghosts were then pelleted by centrifugation and washed as described above.

Labeling of bovine serum albumin

Bovine serum albumin (1%, w/v) in isotonic phosphate buffer, pH 8.0, was treated with 1—5 mM [^{3}H]TMAS at 37°C for 1—2 h. The protein was precipitated with 10% cold trichloroacetic acid washed with trichloroacetic acid, dissolved in 0.1 M NaOH, and assayed for protein and radioactivity

Gel electrophoresis

Polyacrylamide slab gel electrophoresis (7.5% acrylamide in 0.1% SDS) was used to resolve the membrane proteins. The electrode buffer (pH 8.3) contained 0.02 M Tris 0.19 M glycine and 0.1% SDS, and the gels were stained with Coomassie Blue and periodic acid-Schiff's reagent [20]. The gels were cut manually into 1 mm slices incubated each with 50 μl of 0.1 M NaOH in 3 ml scintillation vials for 60 min at room temperature and 0.3 ml of NCS solubilizer (Nuclear Chicago) was then added and the mixture kept in the dark for 1 h. To each vial 2.7 ml of toluene-based scintillation fluid was added and radioactivity was counted in a scintillation spectrometer.

Determination of protein content, specific radioactivity and free amino groups

Solubilized membranes and other samples were precipitated overnight with 60% ethanol at —20°C in 1 ml Eppendorf microcentrifuge tubes. The precipitated proteins were collected by centrifugation and dissolved in 10 μl of 0.1 M NaOH. Protein was determined by the method of Lowry et al. [21] with bovine serum albumin as a standard. Determination of radioactivity was carried out after 10% trichloroacetic acid precipitation onto 25-mm diameter filter discs (Whatmann GF/C). The precipitated proteins were washed by ethyl ether, heated to dryness and then counted.

Free amine groups were assayed by the ninhydrin method [22].

Trypsin digestion

Labeled erythrocyte ghosts were suspended (10%, v/v) in isotonic phosphate buffer, pH 7.4, containing 0.5 mg trypsin (Worthington) per ml and incubated 10 min at 37°C. Lima bean trypsin inhibitor (Worthington) (0.5 mg per ml reaction mixture) was then added. The membranes were collected by centrifugation at 30000 $\times g$ for 20 min and washed twice with isotonic phosphate buffer, pH 7.4, by centrifugation.

Results

Optimal coditions for labeling with TMAS

pH dependence. Bovine serum albumin and intact erythrocytes were treated with 1.5 mM [^{3}H]TMAS for 70 min, at 37°C in isotonic phosphate buffer at different pH values. Fig. 1 shows that at slightly alkaline pH larger amounts of radioactivity are incorporated into bovine serum albumin as well as into the ghost membrane proteins. Maximal labeling of bovine serum albumin was observed above pH 9.0, indicating that the nucleophilic sites on the proteins have an apparent pK above 8.5. The reaction of TMAS with intact erythrocytes showed a similar dependence on the pH of the suspending medium. Under these conditions no measurable hemolysis was detected and globin, an internal protein, was hardly labeled. We chose pH 8.0 for the labeling reactions as a compromise between the physiological pH of 7.4 and the slightly alkaline conditions allowing maximal labeling.

Saturation of accessible sites. Bovine serum albumin was treated with 1 mM [^{3}H]TMAS in isotonic phosphate buffer, pH 8.0, for up to 4 h, at 37°C. Maximal incorporation of the radioactive label was observed within 80 min, and we therefore chose a standard incubation time of 2 h. To determine the optimal concentration of the probe, bovine serum albumin was labeled with increasing concentrations of [^{3}H]TMAS (0—150 mM) for 2 h, at 37°C in isotonic phosphate buffer, pH 8.0. As shown in Fig. 2, maximal incorporation was achieved at about 0.2 mM TMAS. This corresponds to 10—30 mol of TMAS per one protein amino group of bovine serum albumin. We have, therefore, chosen to use between 1 and 4 mM of TMAS to saturate all accessible sites. It was also noted that saturation of protein amino groups as measured by the ninhydrin reaction

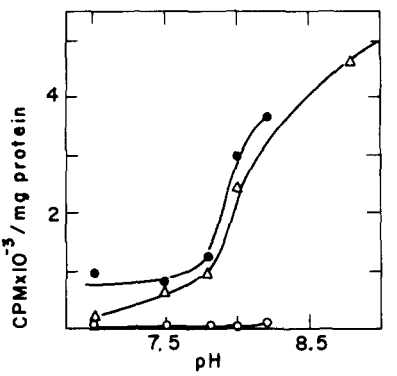

Fig. 1. pH dependence of the reaction of [^{3}H]TMAS with bovine serum albumin, ghost membranes and globin, prepared from prelabeled intact erythrocytes. The various preparations were incubated for 70 min at 37°C. Extent of labeling with bovine serum albumin (Δ———Δ), ghost membranes (●———●) and globin (○———○) prepared from prelabeled intact erythrocytes.

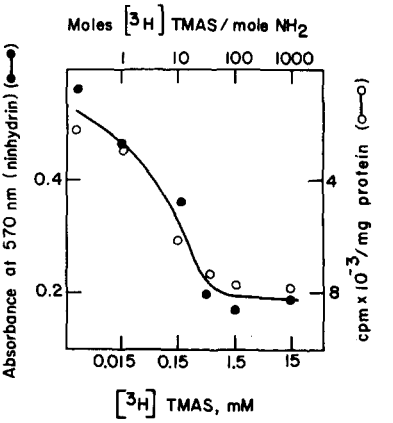

Fig. 2. Reaction of bovine serum albumin with increasing concentrations of [^{3}H]TMAS. Various concentrations of [^{3}H]TMAS (80 mCi/mmol) were incubated with bovine serum albumin for 2 h at 37° C. Incorporation of radioactivity (○———○) and disappearance of ninhydrin-reactive groups (●———●) is plotted as a function of the concentration of TMAS per mol of protein amino groups.

and incorporation of ^3H label occurred in a concurrent manner, indicating that the TMAS reacted primarily with free amino groups (Fig. 2). Under these conditions TMAS appears highly impermeable into intact erythrocytes.

Identification of labeled proteins of erythrocyte membranes

The membrane proteins labeled with [^3H]TMAS were identified by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The electrophoretic pattern of the labeled proteins from ghost membranes represents all the sites that are accessible to the reagent whether they are localized on the outer or inner surface. On the other hand, the labeled proteins derived from intact cells represent primarily outer membrane labeling. Fig. 3b shows the electrophoretic pattern of the labeled ghost proteins which contains a prominent band of apparent molecular weight of 200 000 which may be identical to spectrin. It was also noted that all the ghost proteins visualized by Coomassie Brilliant Blue staining were also labeled by TMAS, although the pattern of radioactive distribution was different. On the other hand, fewer proteins were labeled with TMAS in the erythrocytes and the 200 000 protein was missing (Fig. 3c). The major

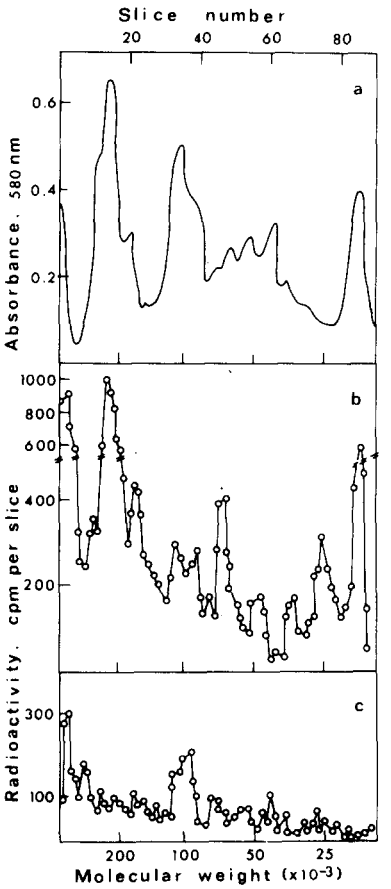

Fig. 3. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of [^3H]TMAS labeled membrane proteins. Densitometric trace of Coomassie Blue staining (a), protein pattern from labeled erythrocyte ghosts (b) and intact erythrocytes (c).

radioactive polypeptide band observed had an apparent molecular weight of 90 000 (approx. 60% of total radioactivity) and may correspond to band 3 or periodic acid-Schiff's reagent band 1 which are not resolved on these gels [23]. Periodic acid-Schiff's reagent band 1 and band 3 were resolved on 9% acrylamide gels [24]; most of the radioactivity was found to coincide with band 3 while a minor fraction overlapped the periodic acid-Schiff's reagent-stained band. In addition, a minor fraction of radioactivity was observed in bands having an apparent molecular weight between 60 000 and 50 000. The electrophoretic pattern observed was independent of the time of incubation or reagent concentration. About 60—70% of the radioactivity could be removed by trypsinization of the labeled erythrocyte ghost membranes while about 30—35% of the radioactivity was extracted with chloroform/methanol mixture. This indicates the TMAS reacted with amino groups of lipids in addition to those of proteins.

Discussion

The present studies illustrate the use of the cationic reagent *N,N,N*,trimethylamino-β-alanyl-*N*-hydroxysuccinimidoester for the labeling of erythrocyte membrane proteins. TMAS reacts with membranes or soluble proteins to form acid-stable addition products which are not dissociated upon reduction with mercaptoethanol. Hence, identification of labeled proteins by sodium dodecyl sulfate polyacrylamide gel electrophoresis, can be achieved. The reaction of TMAS with membranes can be performed at physiological conditions where no detectable hemolysis of erythrocytes was observed.

It appears that TMAS is an amino reactive reagent. This is suggested from the pH dependency of radioactive label incorporation into erythrocyte membranes, which indicates that protein amino acid residues with basic p*K* (approx. 8.0) values become modified (Fig. 1). Moreover, close correlation between the level of TMAS incorporation of the label into proteins and the blocking of accessible amino groups was found (Fig. 2).

TMAS is a cationic reagent and differs from other amino reactive probes [14, 25,26] which are anionic at physiological pH values. The cationic nature of TMAS is expected to cause minimal distortion of membrane structure since the reaction of TMAS results in the displacement of cationic primary ammonium ions of the membrane component by a cationic quaternary ammonium ion attached to a small size backbone.

Application of TMAS labeling for topographical localization of membranal proteins rests upon its inability to penetrate into cell membrane. Indeed, the radioactivity found in globin extracted from intact erythrocytes which were prelabeled with TMAS was less than 1% or the label found in globin from lysed cells. Moreover, spectrin which is localized at the inner surface of the erythrocyte [27] was not labeled in intact cells but is readily labeled in ghost membranes (Fig. 3).

Upon treatment of erythrocyte ghosts with radioactive TMAS all the proteins which are visualized by Coomassie Brilliant Blue staining on sodium dodecyl sulfate polyacrylamide gels become labeled. On the other hand, upon labeling of intact erythrocytes most of the radioactivity migrates with a 90 000 pro-

tein band. Similar patterns of labeled proteins were reported with other amino reactive surface reagents [11,13,14,25,26] and with lactoperoxidase iodination of tyrosine and histidine residues [1—4] although the extent of labeling of certain polypeptides was different depending on the reagent used. This could indicate that in the outer surface of the erythrocytes the 90 000 protein is the major polypeptide component [5] and the one containing the largest number of accessible primary amino groups, as well as tyrosyl residues.

The agreement between the results obtained with anionic and cationic amino reactive probes indicates that the nature of the reactive group rather than the ionic character of the reagent (i.e., anionic or cationic) determine the pattern of labeled exterior polypeptides observed in erythrocytes. In addition it proves the applicability of TMAS as a surface protein reagent. However, one would expect that a considerable effect of the cationic reagents such as TMAS will specifically label some proteins of the excitable membranes, for example those of cholinergic cells. In these cells, low molecular weight "cationic" ligands are thought to play a major role in triggering the excitable membrane. Indeed, we have recently demonstrated differences in the labeling pattern of some acidic proteins from mouse neuroblastoma cells when [^{3}H]TMAS was compared to [^{125}I]lactoperoxidase as the exterior surface membrane reagent (ref. 28 and Zisapel and Littauer, in preparation).

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