A MEMBRANE FILTER ASSAY FOR PROTEIN SULFHYDRYL GROUPS

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Received May 27, 1971

SUMMARY: The adduct formed between ^{14}C-mercuribenzoate and protein SH groups is retained on nitrocellulose filters.

pMercuribenzoate (PMB) has found wide usage in the study of protein sulfhydryl (SH) groups. The formation of PMB-protein SH adducts can be determined spectrophotometrically (1) or by gel filtration of ^{14}C-PMB-protein adducts (2). These procedures have certain inherent difficulties; the spectrophotometric assay cannot be used in the presence of substrates or cofactors which absorb in the UV. The gel filtration procedure is cumbersome for multiple samples and requires a certain length of time for the unreacted ^{14}C-PMB to resolve from the protein making short time kinetic determinations of rapidly reacting SH groups difficult to carry out. We will describe a simple and relatively rapid method for the assay of ^{14}C-PMB bound to protein SH groups. The procedure makes use of the high affinity of nitrocellulose membrane filters for many proteins (3) combined with a low affinity for the ^{14}C-PMB.

MATERIALS AND METHODS:

Azotobacter vinelandii RNA polymerase was purified by a modification of previously published methods and the enzyme is homogeneous (4). Other proteins used were obtained from Sigma Biochemicals or Calbiochem.

Nitrocellulose membrane filters were obtained from Schleicher and Schuell or Matheson and Higgins. The filters (pore size 0.45μ, 25 mm diameter) were soaked in wash buffer for at least 30 mins at room temperature prior to use.

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^{14}C-PMB was a product of Calatomic and was used at a specific activity of 1 x 10^7 cpm/μmole. Radioactivity was determined in a liquid scintillation spectrometer using a toluene-Liquifluor (New England Nuclear) mixture.

RESULTS:

The SH groups of proteins may exist as different reactive classes. Denaturing the protein with urea or with a detergent such as sodium dodecyl

TABLE 1

RETENTION OF THE ^{14}C-PMB-POLYMERASE ADDUCT ON NITROCELLULOSE FILTERS

Filtration Conditions	Wash Buffer	^{14}C-PMB Retained (pmoles)
^{14}C-PMB-Polymerase (0.2 ml)		
+ 2 ml 0.02 M Tris-0.05 M NaCl	Low salt	790
+ 2 ml 0.02 M Tris-1.0 M NaCl	High salt	832
+ 2 ml 8 M urea	Low salt	802
+ 2 ml 0.1% SDS	Low salt	26
+ 2 ml 0.05 M mercaptoethylamine	Low salt	2

The reaction mixture for preparing the ^{14}C-PMB-Polymerase adduct contained (4 ml volume): 80 mM Tris buffer, pH 7.8, 4 mM $MgCl_2$, 260 μg RNA polymerase holoenzyme (28 pmoles protomer per 0.2 ml aliquot) and 7.5 x 10^{-6} M ^{14}C-PMB (1500 pmoles/0.2 ml aliquot). The mixture was incubated for 10 minutes at 37° and 0.2 ml aliquots were added to the mixtures indicated in the Table and incubated for an additional 5 minutes at 37° prior to filtration. The filters were washed with about 10 ml of the wash buffer following adsorption of the protein. Low salt - 0.02 M Tris buffer, pH 7.8 - 0.05 M NaCl; High salt = 0.02 M Tris buffer, pH 7.8 - 1.0 M NaCl.

sulfate (SDS) can expose buried or relatively unreactive SH groups. Some proteins such as RNA polymerase undergo salt-dependent monomer-dimer transitions (5,6) and the presence of a high salt concentration during the assay may render certain SH groups available for titration. The results presented in Table 1 show that neither 1 M NaCl nor 7.2 M urea has any pronounced effect on the binding of the ^{14}C-PMB-polymerase adduct to the filter when compared with the assay using the standard wash conditions (0.02 M Tris buffer, pH 7.8 - 0.05 M NaCl). However dilution of the prelabeled enzyme into 0.1% SDS markedly reduces the amount of labeled protein which binds to the nitrocellulose membrane and therefore this denaturant cannot be used in this procedure. Incubation of the ^{14}C-PMB-protein with a sulfhydryl reagent such as mercaptoethylamine releases the ^{14}C-PMB from the protein as a soluble mercaptide which does not bind to the filter. Blank values for the amount of ^{14}C-PMB bound to the nitrocellulose filter in the absence of added protein are generally less than 0.1% of the input reagent and does not constitute a problem.

The linear relationship between the amount of ^{14}C-PMB retained on the nitrocellulose filter as a function of the amount of protein titrated is shown in Figure 1. The assay for the SH groups of the Azotobacter vinelandii RNA polymerase is linear between 5µg and 50µg of protein (the largest amount tested). Below 5µg protein per assay the amount of ^{14}C-PMB retained is less than expected perhaps due to loss of protein on the surface of the test tube or pipettes used in the experiment even though 5% glycerol was included during the assay and in the wash buffer. The protein binding capacity of the nitrocellulose filter was determined using the ^{14}C-PMB-bovine serum albumin adduct, up to 300 µg of protein can adsorb to a 25 mm filter.

To acertain the general applicability of the procedure several other proteins were tested for their ability to bind to the nitrocellulose filters. The results presented in Table 2 indicate that the proteins assayed bind

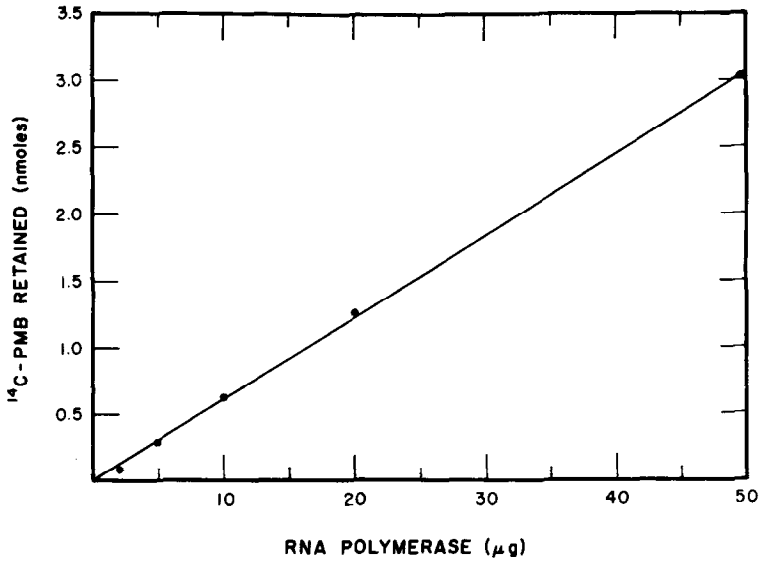

Figure 1

Effect of increasing protein concentration on the amount of ^{14}C-PMB retained. The reactions contained (final volume 0.25 ml): 80 mM Tris.HCl, pH 7.8, 4 mM $MgCl_2$, 0.5 M KCl, 2 x 10^{-5} M ^{14}C-PMB, 5% glycerol and RNA polymerase as indicated. The mixtures were incubated for 20 minutes at 0^{o}. After addition of 2 ml cold 0.02 M Tris buffer, pH 7.8 - 0.05 M NaCl-5% glycerol the samples were filtered onto nitrocellulose filters and washed with 10 ml of the dilution buffer. The blank value (no polymerase) was 3 pmoles ^{14}C-PMB and has been subtracted from the data plotted.

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to the filter and that the amount of ^{14}C-PMB retained corresponds to the expected number of titratable SH groups of the respective proteins. Yeast alcohol dehydrogenase (M = 150,000) shows 8 titratable SH groups per molecule (7), RNA polymerase (M = 470,000) about 30 (5) and horse heart cytochrome C (M = 12,400) none (2). The retention of the cytochrome C on the filter is inferred from the pink color remaining on the filter after the buffer wash. Bovine serum albumin (8) shows 0.15 SH groups per molecule when the assay is run in the absence of urea and 0.6 SH groups when carried out in the presence of 7 M urea. The ^{14}C-PMB adducts of β lactoglobulin, ovalbumin and rabbit muscle aldolase were also retained on the nitrocellulose filters,

TABLE 2

Retention of ^{14}C-PMB-Protein Adducts

on Nitrocellulose Filters

Proteins	pmoles	Urea	^{14}C-PMB Retained (pmoles)
Cytochrome C (horse heart)	1200	-	3
	1200	+	4
Bovine serum albumin	450	-	64
	450	+	274
Alcohol dehydrogenase (yeast)	155	-	1290
	155	+	1105
RNA polymerase	30	-	895
	30	+	1006
Omit protein		-	4
		+	5
Glutathione	10,000	-	1

The reactions contained (final volume 0.3 ml): 100 mM Tris buffer, pH 7.8, 4 x 10^{-5} M ^{14}C-PMB and the proteins indicated. Where indicated 6 M urea was present during the incubation. After 10 minutes at 37°, 2 ml of 0.02 M Tris.HCl, pH 7.8 - 0.05 M NaCl was added and the samples were filtered onto a nitrocellulose filter and washed with 10 ml of the Tris-NaCl buffer. The "omit protein" blank values have been subtracted from the protein data.

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the presence of 1 M NaCl in the wash buffer did not affect the amount of labelled protein retained. The adduct formed between ^{14}C-PMB and the tri-peptide, glutathione (glu-cysSH-gly) was not retained by the nitrocellulose filter under our assay conditions. We do not know what minimum size or

amino acid composition is necessary for adsorption of proteins to nitrocellulose filters.

DISCUSSION:

Because of its specificity PMB has been a reagent of choice in the determination of protein SH groups. The method described in this communication avoids the disadvantages inherent in the spectrophotometric procedure (1) or the gel filtration method (2). The high affinity of nitrocellulose membrane filters for many proteins (3) combined with a low affinity for the ^{14}C-PMB allows the quantitation of SH groups using small amounts of protein, on the order of 5-20 μg per assay. It is possible to study the kinetics of mercaptide formation at relatively short incubation times since the adsorption of the ^{14}C-PMB-protein adduct is rapid and apparently quantitative. The presence of other ligands which could interfere with the spectrophotometric assay does not directly affect the filter assay described. As an example it is possible to show that while the presence of the ribonucleoside triphosphate substrates does not affect the rate of reaction of ^{14}C-PMB with the Azotobacter RNA polymerase, the preformed polymerase-d(A-T) complex shows kinetics of adduct formation indicative of a protected class of enzyme SH groups (9).

ACKNOWLEDGMENTS:

This work was supported by Grant GM 12326 from the National Institutes of Health and Grant NgR 05-003-020 from the National Aeronautics and Space Administration.

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