Anti-Nitroxide Immunoglobulin G: Analysis of Antibody Specificity and Their Application as Probes for Spin-Labeled Proteins†

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ABSTRACT: Antibodies have been elicited to the nitroxide spin-label 4-maleimido-2,2,6,6-tetramethylpiperidinyl-1-oxy conjugated, via protein sulfhydryl groups, to bovine serum albumin. Antibody–hapten cross-reactivity was demonstrated by double immunodiffusion and by a broadening of the nitroxide electron paramagnetic resonance spectrum. The specificity of the antibodies with respect to hapten structure was examined by means of a simple filter binding assay. Under these conditions, antibodies were shown to distinguish between the nitroxide and hydroxylamine derivatives and between spin-labels comprising either five- or six-membered ring structures. In addition, protein-bound nitroxide spin-labels were detected at the nanogram level by immunoblotting. By use of this method, the specificity of the antibody–hapten reaction predicted by the filter binding assay procedure was utilized to differentially detect various types of bound spin-label. Finally, antibodies were used to identify protein-bound nitroxide spin-label of protein fractionated by gel electrophoresis.

Covalent incorporation of stable nitroxide spin-labels into isolated proteins and heterogeneous, membrane systems has been used to study changes in protein conformation and membrane architecture [for reviews, see Berliner (1976) and Marsh (1981)]. However, a major limitation of the procedure used to achieve covalent nitroxide incorporation, particularly with membrane systems, is the difficulty in determining the distribution of the spin-labels among the various protein components. Indirect methods have been most frequently utilized that involve the substitution of a radioactive analogue, such as *N*-[^{14}C]ethylmaleimide, for the corresponding nitroxide probe (4-maleimido-2,2,6,6-tetramethylpiperidinyl-1-oxy). However, both steric and polarity effects could result in altered substitution patterns.

Proteins immobilized on nitrocellulose membranes can be specifically detected by incubation with labeled antibodies in a version of the technique called immunoblotting (Towbin et al., 1979; Gershoni & Palade, 1983). Theoretically, this same blotting technique could be used to follow a ligand labeling pattern where the antibodies elicited against the ligand are used to detect the modified proteins. McConnell and coworkers (Humphries & McConnell, 1976) demonstrated that antibodies can be elicited against a nitroxide spin-label and studied the effect of these antibodies on the electron paramagnetic resonance (EPR)[1] spectrum of immobilized nitroxide spin-labels associated with membrane components.

In this study, we report on the properties of antibodies directed against a sulfhydryl-specific nitroxide spin-label and demonstrate that they provide an exquisitely sensitive probe to detect and identify proteins carrying a nitroxide spin-label.

Materials and Methods

Materials

Bovine serum albumin (fraction V, essentially fatty acid free) and rabbit serum albumin (fraction V) were purchased from Sigma. TEMPO, 4-hydroxyl-TEMPO, 4-maleimido-TEMPO, and 3-maleimido-PROXYL were obtained from Aldrich Chemical Co. Nitrocellulose filters (2.4 cm, HAWP, 0.45 μm) were purchased from Millipore. DEAE-cellulose 52 and CM-cellulose 52 were obtained from Whatman. Complete and incomplete Freund's adjuvant was purchased from Miles Laboratories. The Immuno-Blot assay kit containing goat anti-rabbit IgG–horseradish peroxidase conjugate and nitrocellulose membrane (0.45 μm) were purchased from Bio-Rad. L-[^{35}S]Cysteine (1050 Ci/mmol) was obtained from Amersham.

Methods

Immunization and Antiserum Preparation. Procedures used in the production of antisera to the albumin–4-maleimido-TEMPO conjugate were as described previously by Eichler & Glitz (1974). One milligram of conjugate dissolved in 0.4 mL of 0.1 M NaCl, 0.05 M Tris-HCl, pH 7.5, and 0.1 mM EDTA was mixed with 1.6 mL of complete Freund's adjuvant to form a uniform homogenate. Approximately 0.1 mL of this homogenate was injected into each toe pad of a 3-month-old New Zealand white rabbit. After 2 weeks, the rabbit was injected subcutaneously in the hind quarters with 0.5 mg of conjugate dissolved in 0.1 mL of buffer and mixed with 0.4 mL of incomplete Freund's adjuvant. Four weeks from the time of the initial injection, blood was collected from the ear vein and the serum tested by double diffusion on agar plates (Ouchterlony, 1968). Subsequently, at intervals of 7–8 days, the rabbit was bled from the ear vein, the blood was allowed to clot, and the sera were removed and clarified by centrifugation at 2000*g*. The clarified serum was either processed for preparation of an IgG protein fraction or stored frozen at −70 °C.

Coupling of 4-Maleimido-TEMPO to Bovine Serum Albumin. Ten milligrams of bovine serum albumin was dissolved

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[1] Abbreviations: EPR, electron paramagnetic resonance; TEMPO, 2,2,6,6-tetramethylpiperidinyl-1-oxy; PROXYL, 2,2,5,5-tetramethylpyrrolidinyl-1-oxy; EDTA, ethylenediaminetetraacetic acid; NADH, nicotinamide adenine dinucleotide, reduced form; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Bicine, *N,N*-bis(2-hydroxyethyl)glycine; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; BSA, bovine serum albumin; IgG, immunoglobulin G.

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in 1 mL of sodium borate buffer, pH 9.5, containing 0.3 M NaCl, 1 mM EDTA, and 7 M urea. The buffer was thoroughly degassed by vacuum following which 5 mg of 4-maleimido-TEMPO was added and allowed to mix in the dark at room temperature. After 48 h, the reaction mixture was applied to a Sephadex G-25 column (1.5 × 30 cm) equilibrated against 0.1 M NaCl, 0.05 M Tris-HCl, pH 7.5, and 0.1 mM EDTA. Peak fractions of the void material were pooled and concentrated by vacuum dialysis against this same buffer to a final volume of 1 mL. The number of 4-maleimido-TEMPO residues bound per albumin molecule was estimated in the following manner. The concentration of protein in the conjugate sample was determined by the method of Lowry et al. (1951). The concentration of 4-maleimido-TEMPO was determined from double integration of the EPR spectrum of the conjugate sample using a known concentration of free TEMPO as the standard. The ratio of the molar concentrations of 4-maleimido-TEMPO and protein gave the number of conjugated TEMPO residues per albumin molecule. In a similar manner, 4-maleimido-TEMPO was conjugated to rabbit serum albumin and characterized.

Immunodiffusion. Double-diffusion (Ouchterlony, 1968) agar was made up in 0.1 M NaCl, 0.05 M Tris-HCl, pH 7.5, and 0.1 mM EDTA. Standard antigen solutions were 1 mg/mL unless otherwise specified. Serum was placed in the center well and antigen in the outer wells. Development was allowed to take place overnight at room temperature. The immunodiffusion plates were then washed overnight in 0.9% NaCl, rinsed with distilled water, and dried in a hood after being covered with a moist strip of filter paper. Once the plates were completely dry, the filter paper strip was removed, and the plates were stained by using 0.6% Amido Black made up in destaining solution (5 parts methanol, 5 parts water, and 1 part acetic acid). The plates were washed quickly with distilled water to remove excess stain and destained for 15 min.

Purification of γ-Globulin. Preparation of IgG fraction from whole serum was carried out as described by Palacios et al. (1972). Antiserum (10 mL) was diluted with an equal volume of 0.1 M NaCl, 0.05 M Tris-HCl, pH 7.5, and 0.1 mM EDTA. At 0 °C, 4.52 g of solid ammonium sulfate was added slowly until 40% saturation was achieved. After 30 min, the precipitate was collected by centrifugation at 20000*g* for 15 min. The pellet was dissolved in 4 mL of 0.01 M sodium phosphate, pH 7.2, and 0.015 M NaCl and dialyzed overnight against this same buffer. The dialyzed sample was clarified by centrifugation and applied to a DEAE-CM-cellulose column (1.5 × 10 cm) which contained 5 cm of DEAE-cellulose overlayed with 5 cm of CM-cellulose, both equilibrated against the same buffer, 0.01 M sodium phosphate, pH 7.2, and 0.015 M NaCl. The flow-through fractions were pooled and concentrated by vacuum dialysis against 0.1 M NaCl, 0.05 M Tris-HCl, pH 7.5, and 0.1 mM EDTA.

Filter Binding Assay. The nitrocellulose membrane filter assay technique described by Yarus & Berg (1970) for the study of protein–ligand interactions was used to study hapten–antibody binding. Each assay contained 10 μg of IgG protein and 7.3×10^{-9} M [^{35}S]cysteine-4-maleimido-TEMPO (specific activity 1.3×10^4 cpm/pmol) in 0.25 mL of 0.1 M NaCl, 0.05 M Tris-HCl, pH 7.5, and 0.1 mM EDTA. Samples were incubated at 37 °C for 30 min and transferred to an ice-water bath for 30 min following the protocol of Milstone et al. (1978). Filtrations of 0.2-mL aliquots of each sample were carried out on Millipore glass filter holders. Filters were prewetted in buffer prior to filtration. After sample filtration, the filter was washed 3 times with 1 mL of ice-cold buffer. Filters were dried under a heat lamp and transferred to scintillation vials for counting.

Preparation of Spin-Labeled Nitrate Reductase. Purified NADH:nitrate reductase from *Chlorella vulgaris* (Howard & Solomonson, 1982) was incubated with 2 mM 4-maleimido-TEMPO at a concentration of 1.5 mg/mL in 0.1 M potassium phosphate buffer, pH 7.5. Excess reagents were removed, after >95% loss of NADH:nitrate reductase activity, by passage through a Sephadex G-25 column (0.7 × 16 cm) equilibrated with 50 mM Bicine buffer, pH 7.5, and 0.1 mM EDTA. Peak fractions were located by reduced methyl viologen–nitrate reductase activity which is not affected by sulfhydryl group modification (Solomonson, 1974). The number of TEMPO molecules conjugated to nitrate reductase was deduced by comparison of the optical and EPR spectra of the nitroxide–enzyme complex. Approximately one molecule of 4-maleimido-TEMPO was bound per heme of nitrate reductase.

SDS Gel Electrophoresis. Proteins were separated by SDS–polyacrylamide slab gel electrophoresis on linear gradients of 7.5–15% polyacrylamide by using the buffer system described by Laemmli (1970). Molecular weight standards (Sigma, MW-SDS-200) used were the following: myosin M_r 205 000; β-galactosidase, M_r 116 000; phosphorylase *b*, M_r 97 000; bovine serum albumin, M_r 66 000; ovalbumin, M_r 45 000; and carbonic anhydrase, M_r 29 000.

Immunoblotting. Immunoblot analysis of either proteins transferred from SDS–polyacrylamide gels to nitrocellulose membranes (Towbin et al., 1979; Burnette, 1981) or proteins directly applied to nitrocellulose membranes in a slot-blot apparatus (Schleicher & Schuell) was performed according to the procedures accompanying the Bio-Rad Immuno-Blot-(GAR-HRP) assay kit. In the case of proteins separated by SDS–PAGE, proteins were electrophoretically transferred from the polyacrylamide slab gel to a nitrocellulose membrane with 25 mM Tris, 192 mM glycine, pH 8.3, and 20% (v/v) methanol as the transfer buffer and a voltage of 60 V for 16 h at 4 °C. In the case of direct application of proteins to nitrocellulose, 200 μL of a protein solution was applied to each well of the slot-blot apparatus without vacuum. A vacuum was then applied, and each "slot" was washed with approximately 2 mL of 20 mM Tris and 500 mM NaCl, pH 7.5 (TBS). Excess protein binding sites on the nitrocellulose membranes were blocked by incubation for 1 h at room temperature with TBS containing 3% gelatin. The blocked membranes were incubated with the first antibody or preimmune IgG in TBS–1% gelatin for 2 h. The membranes were rinsed with water and then incubated with two changes of TBS–0.05% Tween-20 followed by one change of TBS for 20 min each. The membranes were then incubated with the second antibody, a 1:2000 dilution of goat anti-rabbit IgG (Bio-Rad) in TBS–1% gelatin, for 1 h. The membranes were rinsed following incubation of the second antibody and subsequently incubated in horseradish peroxidase (HRP) color development medium until blue bands appeared. This development solution was prepared by mixing 30 mg of HRP color development reagent (Bio-Rad), dissolved in 10 mL of methanol, with 50 mL of TBS containing 40 μL of 30% H_2O_2 immediately prior to use. Color development was terminated by rinsing the membranes with distilled water. The stained membranes were incubated with 50% glycerol for approximately 10 min, blotted dry, and stored under clear plastic film.

EPR Spectra. EPR spectra were recorded with a Varian E109 spectrometer (Varian Instruments Inc., Palo Alto, CA) operating at 9-GHz and 100-kHz modulation frequency.

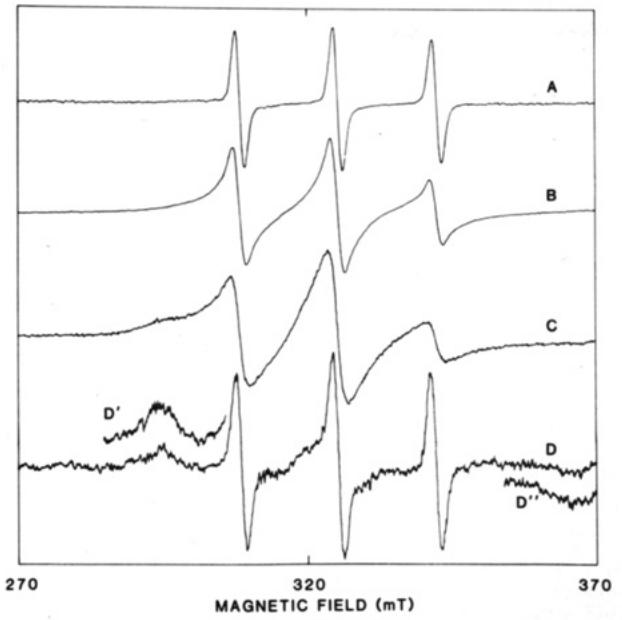

FIGURE 1: EPR spectra of 4-maleimido-TEMPO: (A) 4-maleimido-TEMPO (230 μM) in 50 mM Tris-HCl buffer containing 100 mM NaCl, pH 7.5; (B) 4-maleimido-TEMPO conjugated to bovine serum albumin, (13 mg/mL) in 50 mM Tris-HCl buffer containing 100 mM NaCl, pH 7.5; (C) rabbit serum albumin–4-maleimido-TEMPO conjugate incubated with excess anti-nitroxide IgG fraction to form an immune precipitate (the precipitate was sedimented by centrifugation and resuspended in 50 mM Tris-HCl buffer containing 100 mM NaCl, pH 7.5; (D) cysteine–4-maleimido-TEMPO incubated with anti-nitroxide IgG fraction.

Spectra were routinely obtained at room temperature by using capillary quartz sample tubes under conditions of 14-mW microwave power and a modulation amplitude of 0.063 mT. Concentrations of paramagnetic species were obtained by double integration of the EPR spectra according to the method of Wyard (1965).

RESULTS

Preparation and Characterization of Antigen. Ligand coupled to a protein carrier is an established approach to elicit antiserum to specific ligands (Erlanger, 1980). Bovine serum albumin is most often used as the protein carrier because of its excellent immunogenic properties (Erlanger, 1980; Sela, 1969). In order to effectively couple the nitroxide spin-label 4-maleimido-TEMPO to bovine serum albumin, we took advantage of the relatively high number of titratable sulfhydryl groups present in the albumin molecule. Previously, it was shown that the titration of the albumin thiol groups was dependent not only on the presence of a denaturant such as urea but also on the pH (Axen et al., 1977). For this reason, the sulfhydryl-reactive spin-label was reacted with the albumin carrier in 7 M urea under alkaline conditions to maximize substitution. Under these conditions, maleimide derivatives may also react with other functional groups of amino acids (Smyth et al., 1960, 1964). Approximately 13 residues of nitroxide spin-label were found per albumin molecule. Covalent modification of the accessible groups of bovine serum albumin by 4-maleimido-TEMPO resulted in a significant change in the EPR spectrum of the bound nitroxide (Figure 1), reflecting a decrease in the rotational mobility of the conjugated spin-label. A similar spectrum was obtained for the corresponding rabbit serum albumin–4-maleimido-TEMPO conjugate.

Characterization of Antibodies. Sera were monitored for antibody content by double-diffusion analysis and exhibited cross-reactivity to 4-maleimido-TEMPO coupled to either

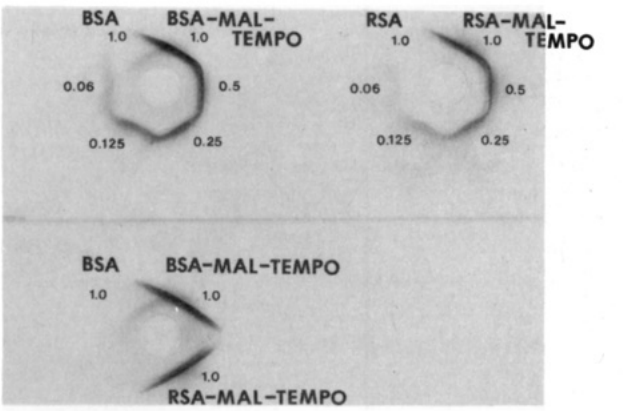

FIGURE 2: Immunodiffusion of anti-nitroxide serum. The center wells contained anti-nitroxide serum collected at 6 weeks from the initial toe pad injection. The peripheral wells contained the indicated concentrations (in milligrams per milliliter) of antigen. BSA, bovine serum albumin; BSA–MAL-TEMPO, 4-maleimido-TEMPO conjugated to bovine serum albumin; RSA, rabbit serum albumin; RSA–MAL-TEMPO, 4-maleimido-TEMPO conjugated to rabbit serum albumin.

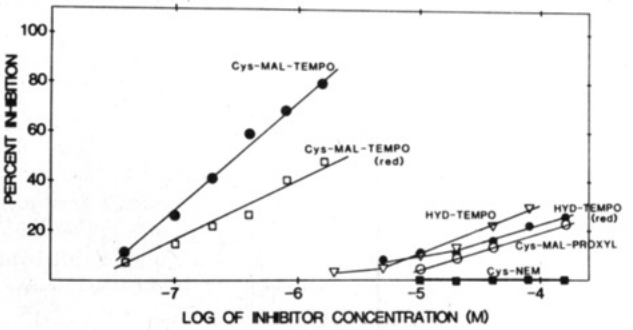

FIGURE 3: Inhibition of binding of [^{35}S]cysteine–4-maleimido-TEMPO to an antibody elicited with 4-maleimido-TEMPO as hapten. Nonradioactive analogues were used as competitive inhibitors at the concentrations indicated. Cys–MAL-TEMPO, cysteine–4-maleimido-TEMPO; Cys–MAL-TEMPO (red), cysteine–4-maleimido-2,2,6,6-tetramethylpiperidinyl-1-hydroxylamine; HYD-TEMPO, 4-hydroxy-TEMPO; HYD-TEMPO (red), 4-hydroxy-2,2,6,6-tetramethylpiperidinyl-1-hydroxylamine; Cys–MAL-PROXYL, cysteine–3-maleimido-2,2,5,5-tetramethylpyrrolidinyl-1-oxy; Cys–NEM, cysteine–*N*-ethylmaleimide.

bovine serum albumin or rabbit serum albumin as shown in Figure 2. Little cross-reactivity was observed against unsubstituted bovine serum albumin, demonstrating that the response was primarily directed against the nitroxide spin-label hapten. Sera collected from the sixth and seventh week after the primary injection were chosen for use in our experiments since later bleedings progressively demonstrated a greater proportional response to the carrier molecule, BSA, with no further significant increase in the average association constant between antibody and hapten. Since it has been previously shown that purification of antibodies to a nitroxide spin-label using affinity chromatography techniques typically results in a loss of binding affinity (Brulet & McConnell, 1976), we chose to purify only the serum immunoglobulin fraction, and this IgG fraction was utilized in all subsequent studies.

To determine the antibody specificity, 4-maleimido-TEMPO was labeled by conjugation with L-[^{35}S]cysteine. This labeled ligand conjugate was used as the probe in the filter binding assays. As shown in Figure 3, the antibodies bind the labeled probe cysteine–4-maleimido-TEMPO with the highest affinity. The concentration of various compounds required to displace half of the bound radioactive probe from the IgG fraction was determined and is summarized in Table I. These antibodies can distinguish the cysteine-conjugated nitroxide spin-label

Table I: Inhibitor Concentration Necessary To Produce 50% Inhibition of Binding of [^{35}S]Cysteine–4-Maleimido-TEMPO to Anti-Nitroxide IgG[a]

inhibitor	concn (M)
cysteine–4-maleimido-TEMPO	0.24
cysteine–4-maleimido-TEMPO (red)[b]	1.7[c]
4-hydroxy-TEMPO	140[c]
4-hydroxy-TEMPO (red)[b]	320[c]
cysteine–3-maleimido-PROXYL	380[c]
cysteine–*N*-ethylmaleimide	ND[d]

[a] Details of the procedure are given under Materials and Methods. [b] (red) specifies the corresponding hydroxylamine derivative. [c] Extrapolated values. [d] No inhibition detected.

from the dithionite-reduced (hydroxylamine), diamagnetic, form of this same structure with almost an order of magnitude difference in the effective concentration required to displace 50% of label. Removal of the maleimide and cysteine groups greatly weakens the binding since 4-hydroxy-TEMPO competes 1000 times less effectively than the cysteine–maleimido-TEMPO conjugate. At this level of recognition, the diamagnetic hydroxylamine derivative of hydroxy-TEMPO is recognized just as effectively as the nonreduced, paramagnetic form. The linkage structure, in the form of cysteine–*N*-ethylmaleimide, did not, by itself, compete with the labeled probe at the concentrations examined. Interestingly, the substitution of a five-membered PROXYL ring structure for the TEMPO moiety in the maleimide–cysteine arm was recognized at an equivalent level as hydroxy-TEMPO alone. Taken together, these results demonstrate that each constituent of the nitroxide spin-label conjugate contributes to the overall recognition process but that the six-membered nitroxide spin-label ring structure, TEMPO, appears to be the most important determinant.

Antibody–hapten interaction could also be demonstrated by EPR spectroscopy. Formation of an immunoprecipitate between the antibodies and the rabbit serum albumin–4-maleimido-TEMPO conjugate resulted in a significant broadening of the nitroxide EPR spectrum (Figure 1C). However, the appearance of a strongly immobilized component could be readily demonstrated by using the cysteine–nitroxide conjugate (Figure 1D). While signals due to excess cysteine–nitroxide are evident, the antibody–hapten conjugate exhibited a separation between the low-(D′) and high- (D″) field hyperfine lines of approximately 7.2 mT ($2T_{zz}$). Incubation of the cysteine–4-maleimido-TEMPO conjugate with increasing concentrations of the IgG fraction resulted in a progressive decrease in the EPR signal amplitude as shown in Figure 4. A plot of the relative signal amplitude vs. [IgG] added (Figure 4B) revealed a rapid, initial decrease in the EPR signal reaching a limiting value at high IgG levels. No change in the EPR spectrum was observed following incubation of the cysteine–nitroxide conjugate with control, preimmune serum.

Detection of Nitroxide Spin-Labeled Proteins. Purified nitrate reductase (Howard & Solomonson, 1982) was treated with 4-maleimido-TEMPO such that one nitroxide spin-label was incorporated per protein subunit. Unreacted nitroxide was removed by passage through a Sephadex G-25 column and the spin-labeled protein applied to nitrocellulose over a 100-fold range of protein concentrations. The nitrocellulose membrane was incubated with unlabeled rabbit anti-spin-label antibodies, washed, and subsequently incubated with peroxidase-labeled goat anti-rabbit antibody. Detection was accomplished by using the peroxidase reaction. As can be seen in Figure 5, the antibodies to spin-label and the immunoblot assay system can be used to detect as little as 0.1 pmol of nitroxide spin-label conjugated to protein, and that detection is absolutely dependent on prior labeling with 4-maleimido-TEMPO. In addition, the level of sensitivity seems to be little affected by the reduction of the nitroxide spin-label to the corresponding hydroxylamine at higher levels of antibody. However, at lower concentrations of antibody, differences were detected. Thus, the highest sensitivity was observed for the six-membered (TEMPO) nitroxide spin-label conjugated to nitrate reductase as either the *N*-ethylmaleimide or the iodoacetamide derivative. This was followed in detection sensitivity by the reduced hy-

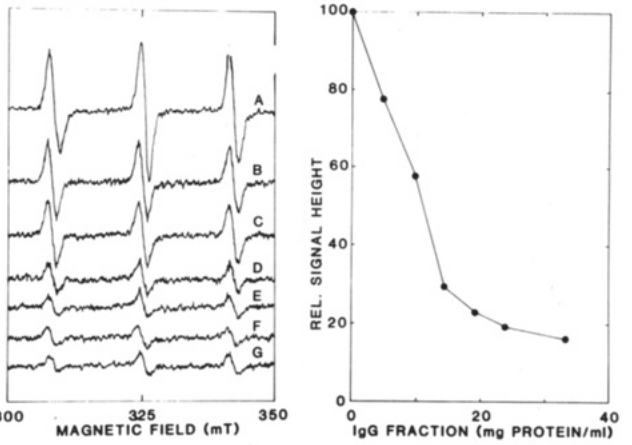

FIGURE 4: Changes in the EPR spectrum of the cysteine–4-maleimido-TEMPO conjugate following titration with the anti-nitroxide IgG fraction. (Left panel) Cysteine–4-maleimido-TEMPO (0.068 μg) was incubated with increasing concentrations of the anti-nitroxide IgG fraction and the EPR spectrum recorded. Spectra correspond to the following IgG concentrations (total protein): (A) 0 μg; (B) 190 μg; (C) 380 μg; (D) 570 μg; (E) 760 μg; (F) 950 μg; (G) 1.33 mg. (Right panel) The relative amplitude of the center line of the nitroxide EPR spectrum is plotted as a function of the concentration of IgG protein added.

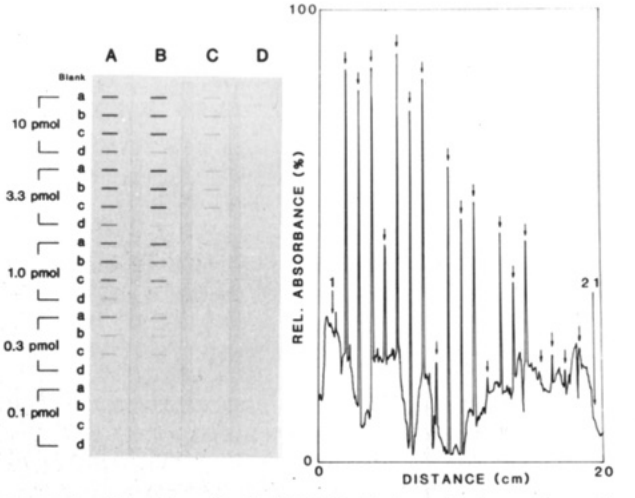

FIGURE 5: Detection of spin-labeled nitrate reductase using anti-nitroxide IgG fraction. Lanes A–D contained the same amounts of either control or spin-labeled nitrate reductase (approximately 10 pmol of spin-label conjugated to 1 μg of nitrate reductase). (Left panel) The quantity of spin-labeled conjugate (in picomoles) applied in each series of rows is indicated along the left-hand margin: (blank) 1 μg of unlabeled control nitrate reductase; (a) nitrate reductase–4-maleimido-TEMPO conjugate; (b) nitrate reductase–4-maleimido-TEMPO conjugate reduced with dithionite to the corresponding hydroxylamine; (c) nitrate reductase–4-(2-iodoacetamido)-TEMPO conjugate; (d) nitrate reductase–3-maleimido-PROXYL conjugate. Lanes A, B, C, and D were incubated with 1:100 dilution, 1:2500 dilution, 1:10 000 dilution, and no anti-nitroxide IgG fraction, respectively (original concentration 32 mg of protein/mL). Other details of the immunoblot procedure are described under Materials and Methods. (Right panel) Desitometer scan of lane B shown in the left panel. Arrows indicate the location of slots. 1 indicates location of the blank, and 21 indicates the location of 0.1 pmol of (d).

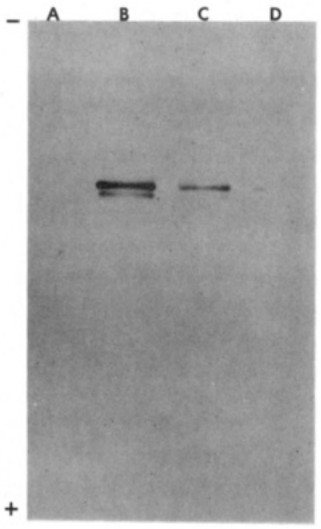

FIGURE 6: Immunoblot detection of spin-labeled nitrate reductase following SDS gel electrophoresis and transfer to nitrocellulose membrane. SDS gel electrophoresis and immunoblot analysis of transferred proteins were as described under Materials and Methods. Samples applied to the SDS gels were as follows: (A) 330 ng of control (unlabeled) nitrate reductase; (B) 330 ng of nitrate reductase–4-maleimido-TEMPO conjugate; (C) 100 ng of nitrate reductase–4-maleimido-TEMPO conjugate; (D) 33 ng of nitrate reductase–4-maleimido-TEMPO conjugate. 100 ng of spin-labeled nitrate reductase contains approximately 1 pmol of 4-maleimido-TEMPO. The major band corresponds to a protein molecular weight of about 100000. A minor band is also observed at the highest protein concentration. This is associated with nitrate reductase as determined by peptide mapping (Howard & Solomonson, 1982) and may reflect microheterogeneity of the carboxy terminus of the enzyme.

droxylamine six-membered derivative and finally by the five-membered (PROXYL) nitroxide spin-label.

The antibodies to the nitroxide spin-label were also used to detect labeled protein after fractionation by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Since the nonreduced or reduced nitroxide spin-labeled protein can be recognized by immunoblotting techniques, we heat-denaturated spin-labeled nitrate reductase in the presence of sodium dodecyl sulfate and β-mercaptoethanol prior to loading them onto the polyacrylamide gel. Following electrophoresis and transfer to nitrocellulose membranes, the labeled protein was detected as described for the immunoblots. As shown in Figure 6, antibodies directed against the spin-label can be used to detect as little as 1 pmol of nitroxide-labeled protein applied to the gel. The apparent difference in sensitivity between the immunoblots and polyacrylamide gel patterns may reflect the efficiency of protein transfer from SDS gels to the nitrocellulose membrane (Gershoni & Palade, 1983).

Discussion

McConnell and co-workers originally demonstrated that antibodies could be elicited against a nitroxide spin-label using spin-labeled analogues of iodoacetamide conjugated to either hemocyanin (Humphries & McConnell, 1976) or L-α-dipalmitoylphosphatidylethanolamine (Brulet & McConnell, 1976) with binding affinities on the order of 10^6 L/mol (Rey & McConnell, 1976). The purified antibodies exhibited nearly equal affinities for both the corresponding diamagnetic hydroxylamine and closely related nitroxide derivatives. The relatively low affinities and selectivity were presumably a consequence of the IgG purification procedure utilized (Brulet & McConnell, 1976).

In the current experiments, we have obtained a significantly higher estimate for antibody affinity and provided a description of the structural factors that influence antibody–hapten interaction. In addition, we have extended the work to include the application of immunoblotting techniques which demonstrate that antibodies to a nitroxide spin-label can detect a monosubstituted labeled protein at the nanogram level. This provides a highly specific and extremely sensitive procedure to detect spin-labeled proteins. In developing this method, we have shown that the extent of antibody dilution can be utilized to alter the sensitivity and therefore the apparent specificity of the immunoblot procedure. At relatively high antibody concentrations, the detection of the nitroxide spin-labeled protein was not significantly affected by prior reduction of the nitroxide spin-label to the hydroxylamine. Moreover, at this concentration of antibody, even the protein spin-labeled with the five-membered PROXYL derivative was detected, although to a lesser extent. However, at the higher antibody dilutions, significant differences between the levels of detection were observed. Therefore, in biochemical experiments, the concentration of the anti-nitroxide antibody can be varied to affect the apparent specificity. This variation can be predicted from the results obtained from the filter binding experiments which measure the apparent affinity for various ligands. For example, in experiments where the spin-label may be internalized, and therefore possibly reduced, this method would permit detection independent of the presence or absence of an EPR signal. Moreover, the level of sensitivity of the immunoblot procedure far exceeds that which can be approached by EPR spectroscopy.

We were also able to demonstrate that the antibodies to the nitroxide could be used to specifically identify labeled proteins after fractionation by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. This approach should be particularly advantageous for detecting and identifying nitroxide-labeled proteins in multicomponent or membrane systems.

References

Axen, R., Carlsson, J., & Drecin, H. (1977) in *Thioprophy-Sepharose 6B*, Pharmacia Bulletin, Pharmacia Chemicals, Piscataway, NJ.

Berliner, L. J. (1976) *Spin Labelling. Theory and Applications*, Academic Press, New York.

Brulet, P., & McConnell, H. M. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 2977–2981.

Burnette, W. N. (1981) *Anal. Biochem. 112*, 195–203.

Eichler, D. C., & Glitz, D. G. (1974) *Biochim. Biophys. Acta 335*, 303–317.

Erlanger, B. (1980) *Methods Enzymol. 70*, 85.

Gershoni, J. M., & Palade, G. E. (1983) *Anal. Biochem. 131*, 1–15.

Howard, W. D., & Solomonson, L. P. (1982) *J. Biol. Chem. 257*, 10243–10250.

Humphries, G. M. K., & McConnell, H. M. (1976) *Biophys. J. 16*, 275–277.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Lowry, O. H., Rosenbrough, N. J., Farr, A. C., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Marsh, D. (1981) *Mol. Biol. Biochem. Biophys. 31*, 51–142.

Milstone, D. S., Vold, B. S., Glitz, D. G., & Shutt, N. (1978) *Nucleic Acids Res. 5*, 3439–3455.

Ouchterlony, O. (1968) *Handbook of Immunodiffusion and Immunoelectrophoresis*, Ann Arbor–Humphrey Science Publishers, Ann Arbor, MI.

Palacios, R., Palmiter, R. D., & Schimke, R. T. (1972) *J. Biol. Chem. 247*, 2316–2321.

Rey, P., & McConnell, H. M. (1976) *Biochem. Biophys. Res. Commun. 73*, 248–254.

Sela, M. (1969) *Science (Washington, D.C.) 166*, 1365–1373.

Smyth, D. G., Nagamatsu, A., & Fruton, J. S. (1960) *J. Am. Chem. Soc. 82*, 4600–4604.
Smyth, D. G., Blumenfeld, O. O., & Konigsberg, W. (1964) *Biochem. J. 91*, 589–595.
Solomonson, L. P. (1974) *Biochim. Biophys. Acta 334*, 297–308.
Solomonson, L. P., & Vennesland, B. (1972) *Biochim. Biophys. Acta 267*, 544–557.
Towbin, H., Staehelin, T., & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350–4354.
Wyard, S. J. (1965) *J. Sci. Instrum. 42*, 768–769.
Yarus, M., & Berg, P. (1970) *Anal. Biochem. 35*, 450–465.

Solubility and Structure of Domains of Chicken Erythrocyte Chromatin Containing Transcriptionally Competent and Inactive Genes

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ABSTRACT: Chromatin generated by micrococcal nuclease digestion of erythrocyte nuclei can be fractionated into two pools of differing solubility in solvents containing 0.15–0.25 M NaCl. A fixed percentage of the chromatin is soluble under these conditions, independent of the average size of the DNA in the unfractionated chromatin. Chromatin containing particular gene sequences is also distributed between soluble and insoluble fractions in a way that is independent of the average size of the starting material. However, the actual percentage of gene copies present in each fraction is not necessarily the same as for bulk chromatin. The transcriptionally active chicken erythrocyte adult β-globin gene is more soluble than the bulk, while the ovalbumin gene in the same tissue is less soluble. These differences do not appear to be related to variations in content of RNA, core histones, or two classes of non-histone proteins. Instead, we find that the soluble chromatin pool is somewhat depleted in histones H1 and H5 and contains lower molecular weight DNA than precipitable chromatin. The soluble fraction can be made insoluble by addition of H1. If the precipitable chromatin fraction is redigested to reduce its size and then recombined with the soluble fraction and reprecipitated, the distribution of globin gene is randomized. The results suggest that the partitioning of chromatin into soluble and insoluble pools in 0.15–0.25 M NaCl arises from redistribution of a limiting amount of histones H1 and H5 to the chromatin fractions containing the longest DNA.

Numerous attempts have been made to fragment chromatin and to separate the product into fractions differing in physical properties in a way that might be correlated with gene activity (Gottesfeld, 1977; Pederson, 1978; Nasser & McCarthy, 1975; Bonner et al., 1975). A number of procedures have made use of mild digestion with nucleases that preferentially attack and release transcriptionally active chromatin. Subsequent fractionation then depends upon some combination of differences in the size and solubility of the active and inactive material.

Recent studies of the enhanced nuclease sensitivity of chromatin near transcriptionally active genes suggest that perturbations in chromatin structure can involve not only the genes themselves but also domains at least several kilobases in length surrounding them (Stalder et al., 1980; Flint & Weintraub, 1977; Wood & Felsenfeld, 1982; Weintraub & Groudine, 1976). this raises the question whether there may be some distinguishable compositional modification of these domains in vivo that would also affect their solubility. The earlier nuclease digestion studies mentioned above unfortunately do not address this question directly, since no attempt was made to separate the size-dependent effects on solubility and yield from effects that are independent of size. A recent exception is the work of Fulmer & Bloomfield (1981), who found that gentle micrococcal nuclease digestion of chicken erythrocyte nuclei yields two populations of chromatin differing in their solubility in 0.15 M NaCl. They deduced that the two populations were apparently not in a precursor–product relationship to each other, since they were released and digested at characteristically different rates at two different temperatures.

In this paper, an attempt is made to determine the physical basis of these solubility properties by studying the solubility behavior of chicken erythrocyte chromatin as a function of salt concentration, molecular weight, and concentration. For all of the chromatin preparations examined, the salting-out behavior does not depend upon the average molecular weight of the starting material or the starting concentration: At salt concentrations below 80 mM, most (80–95%) of the chromatin is soluble; at salt concentrations between 175 and 250 mM, for chromatin preparations varying widely in concentration and starting DNA molecular weight, a fixed fraction (about 65%) of this soluble material precipitates. When fractions are tested for the abundance of the adult β-globin gene and ovalbumin gene, it is found that the former is concentrated in the soluble fraction and the latter in the insoluble fraction. Again, these distributions are quantitatively independent of the molecular weight of DNA in the starting chromatin preparation.

These differences in solubility properties are not correlated with the content of RNA, core histones, or two distinct classes of non-histone proteins. However, the average molecular

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