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Short Note

Elimination of non-specific binding in Western blots from non-reducing gels

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Summary

The reaction of some antibodies with Western blots of protein shows strong non-specific binding especially at a region that corresponds to about 70–90 kDa. This binding is independent of protein concentration. Further analysis indicated that the factor responsible for the non-specific binding is 2-mercaptoethanol in the gel sample buffer. Gel electrophoresis of total tissue homogenates in the absence of this reducing agent resulted in dramatic elimination of the non-specific background binding without affecting the mobility of the two proteins we studied.

Key words: Electrophoresis; Cytochrome P450

Introduction

Immunological probing of Western blots of protein is a powerful means to study the expression and regulation of specific proteins. In this technique proteins from polyacrylamide gels are transferred to a nitrocellulose or nylon membrane; the membrane is reacted first with a specific antibody, and then with a labelled secondary antibody or protein A [1]. The intensity of the label associated with a specific band is taken as a measure of the quantity of the specific protein(s) recognized by the first antibody.

In our use of this technique some antibodies give strong bands of non-specific binding and a high background. Here we report that these non-specific reactions can be eliminated by running the protein sample on the gel without the reducing agent 2-mercaptoethanol that is usually included in the gel sample buffer.

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Materials and Methods

Enzymes and antibodies

The two enzymes used in this work, adrenodoxin and cytochrome P450scc, were purified from bovine adrenal cortex [2]. Both the anti-bovine-adrenodoxin antibody [2] and the anti-rat P450scc antibody [3] were generated in rabbits. The antisera were used without any purification.

Electrophoresis and blotting conditions

In our studies we electrophorese generally 20–40 μg of total tissue (liver, adrenal, ovary) or cell homogenate in a single well of gel. We do not perform cell fractionation as this results in differential recovery of some enzymes [2]. The purified standards or homogenate samples are heated for 2 min at 100 °C in gel sample buffer (final concentrations: 50 mM Tris-HCl, pH 6.8, 1% sodium dodecyl sulfate, 10% glycerol, with or without 10% 2-mercaptoethanol), and electrophoresed in 8.5% (for cytochrome P450) or 14% (for adrenodoxin) polyacrylamide gel with 0.1% sodium dodecyl sulfate as previously described [4]. After electrophoresis the gel is incubated for 30 min in transfer buffer (25 mM Tris, 192 mM glycine, and 20% methanol) and the proteins are transferred to nitrocellulose by electrophoresis in the same buffer at a constant voltage of 70 V at 4 °C for 1.5 h in a Bio-Rad Trans-Blot apparatus.

Antibody reactions

After transfer the blots are washed with 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.05% Tween-20 (TBST), and stored in this buffer at 4 °C overnight. All subsequent incubations and washes are carried out at room temperature with gentle shaking. The blots are first incubated with 1% bovine serum albumin (Sigma A-7906) in TBST for 1 h to block non-specific binding. The antibody is added at a 1 : 200 dilution to the blocking solution and incubation continued for an additional 1 h. The unbound antibody is removed by washing three times for 5–10 min with TBST. To visualize the specifically bound antibody, the blot is incubated with 0.3 μCi ^{125}I-protein A (7.7 μCi/μg, New England Nuclear) in TBST for 1 h. Unbound ^{125}I-protein A is removed by washing three times with TBST. The blot is then dried and autoradiographed at −70 °C.

Results and Discussion

In our studies of the enzymes of the mitochondrial cytochrome P450 systems some antibodies were observed to produce non-specific binding at a position that corresponds to about 70–90 kDa. An anti-rat-P450scc antibody produced the strongest degree of this non-specific binding and analysis of the gel by densitometric scanning became impossible (Fig. 1, middle panel). This non-specific binding was a result of the antisera and not any of the other constituents of the Western blot method, because under identical conditions another antibody for a different protein,

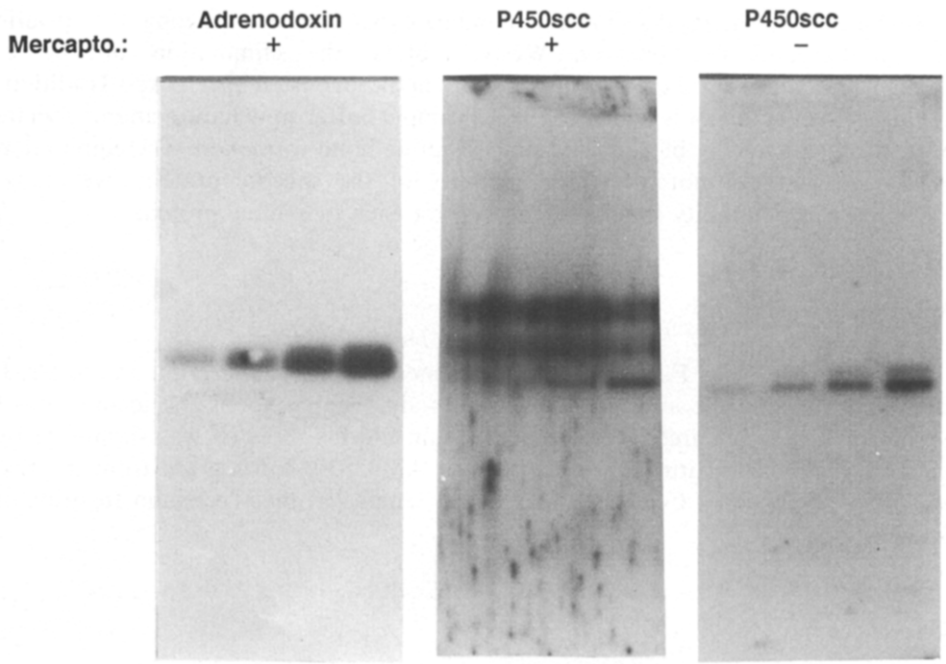

Fig. 1. Elimination of non-specific background binding in Western blots from non-reducing gels. The three panels are autoradiograms of Western blots of three different gels containing either purified adrenodoxin or P450scc. In each gel the four lanes shown included 0.25, 0.5, 1, and 2 pmol of the indicated purified protein. The protein was loaded on the gel in gel sample buffer with (Mercapto.+) or without (Mercapto.–) 2-mercaptoethanol. The blots were reacted with the respective antibodies as described in Methods.

adrenodoxin, produces consistently very clean results with only specific staining of the protein band (Fig. 1). Since the intensity of the non-specific staining was independent of the protein concentration in the lanes of purified protein standards (Fig. 1), we suspected that a constituent of the gel sample buffer is responsible for the non-specific reaction of some antisera. Indeed, elimination of 2-mercaptoethanol from the gel sample buffer resulted in disappearance of the non-specifically stained bands and cleared dramatically the overall background on the whole surface of the blot (Fig. 1). The results shown are for purified proteins and similar results were observed for total tissue homogenates as well. The non-specific binding is not limited to our antisera; similar non-specifically reacting bands have been also noted under reducing conditions with antibodies against some peptide hormones (Dr. F. Kohen, personal communication).

At present we do not know the reason for the 2-mercaptoethanol-dependent non-specific binding by some antisera. The antibody that produced the strongest non-specific staining was generated against a protein eluted from polyacrylamide gels. But this cannot be an explanation as some other antisera also produce similar non-specific staining even though they were generated against antigens that were not gel purified.

In conclusion, the results shown here indicate that in cases of strong non-specific and background reactions on Western blots, the elimination of the 2-mercaptoethanol from the gel sample buffer should be tested as a 'cure'. Traditionally, 2-mercaptoethanol is added to the gel sample buffer to denature and dissociate proteins completely by blocking cysteine disulfide bond formation. The elimination of this reagent did not affect the mobility of the specific proteins we study. However, this possibility should be checked for each individual protein.

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