Techniques for the Analysis of Membrane Proteins

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C.I. RAGAN and R.J. CHERRY

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London New York
CHAPMAN and HALL

First published in 1986 by
Chapman and Hall Ltd,
11 New Fetter Lane, London EC4P 4EE
Published in the USA by
Chapman and Hall
29 West 35th Street, New York NY 10001

Printed in Great Britain at the University Press,
Cambridge

ISBN 0 412 24970 7

British Library Cataloguing in Publication Data

Techniques for the analysis of membrane proteins.
1. Membrane proteins—Analysis
2. Chemistry, Analytic
I. Ragan, C. I. II. Cherry, R. J.
574.87'5 QP552.M44

ISBN 0-412-24970-7

Library of Congress Cataloging in Publication Data

Techniques for the analysis of membrane proteins.
Includes bibliographies and index.
1. Membrane proteins—Analysis. 2. Membrane proteins—Purification. I. Ragan, C. I.
II. Cherry, R. J. [Richard J.] [DNLM:
1. Membrane
Protein—analysis. QU 55 T2547]
QP552.M44T43 1986 574.87'5 86-4183

ISBN 0-412-24970-7

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Preface

A preface should justify the existence of the book it precedes and this is invariably done in scientific texts by reference to the explosive growth of the field since the last such volume appeared. In molecular biology, most fields can be justifiably described as growing explosively, as should be the case for a young and vigorous science, but the study of membrane proteins stands out as one which has taken giant strides in the last few years. Ignorance of the structure and function of membrane proteins at the molecular level was certainly not due to lack of interest but rather was a result of lack of appropriate techniques. It has above all been the development of new experimental methods which has wrenched membrane biochemistry out of what Anthony Martonosi fetchingly called its 'romantic phase' (i.e. lots of ideas and few facts), into an era when the determination of membrane protein structure and mechanism is a reasonable goal.

Membrane proteins are generally classified as peripheral or integral. Peripheral proteins are relatively easily dissociated from membranes by mild treatments whence their study is essentially no different to that of soluble proteins. This book therefore concentrates on integral proteins which are strongly bound to the membrane by hydrophobic interactions with lipids. A crucial step in their study is of necessity the development of methods of solubilization and purification under non-denaturing conditions. A peculiar difficulty with membrane proteins, in particular those which transport substances across membranes, is that their function may not be detectable in solution. An essential part of the investigation of such proteins is therefore the reconstitution of their function by incorporation into artificial lipid membranes. Successful reconstitution into both planar and vesicular membranes has now been reported for a wide range of membrane proteins.

For many years the powerful techniques of peptide sequencing and X-ray crystallography, which have given us a highly detailed picture of the structure and molecular mechanism of many soluble proteins, made little contribution to the study of membrane proteins. Although it is eleven years since the single membrane-spanning sequence of glycophorin was first reported, it was only in 1979 and after much difficulty that the sequence of a larger membrane protein, bacteriorhodopsin, was determined. The hydrophobic nature of membrane proteins still makes direct sequencing a difficult task, but the

advent of recombinant DNA technology and hence sequencing of the corresponding gene is producing dramatic advances in this area.

The method of structure determination by electron microscopy of two-dimensional crystals of membrane proteins was first applied to bacteriorhodopsin in 1975. Although a number of other membrane proteins have been subsequently examined by this method and much insight has been gained as to their molecular architecture, the structures obtained have invariably been of low resolution. Atomic resolution requires three-dimensional crystals suitable for X-ray diffraction studies and the recent success with the bacterial reaction centre is a noteworthy breakthrough. Nevertheless, general application of X-ray crystallography to membrane proteins remains a distant vista and even when achieved will not answer many important questions relating to the organization and interactions occurring within the native biological membrane. Techniques such as freeze–fracture electron microscopy and a variety of methods for studying membrane topology will continue to have an important role, while the immunological approach is making an increasing contribution to the field.

A feature of current investigations of biological macromolecules is an increasing interest in dynamic properties. This is particularly evident in the case of membrane proteins, where both rotational and lateral mobility are a consequence of the fluid nature of the lipid bilayer. The investigation of these motions has required the development of novel techniques and most of the methods currently in use have been developed during the last decade. The study of membrane protein dynamics is important for its functional implications in, for example, electron transport, receptor-mediated responses and various aspects of immunology. In addition, investigation of membrane protein dynamics is contributing significantly to our understanding of interactions between membrane components and between the membrane and cytoskeletal structures.

This book is intended as a guide to the current armoury of techniques for the purification and characterization of membrane proteins and for studying their structure and dynamics. An obvious difficulty for such a volume is that the methods range from those which can be practised in any reasonably well-equipped biochemical laboratory to those involving specialized technical expertise and sophisticated and expensive instrumentation. It has therefore not been practical to try to impose uniformity of treatment but rather we have encouraged contributors to provide a level of experimental detail appropriate to each individual subject. In particular, the authors have tried to convey through their own experience the scope and limitations of their techniques, the kinds of information which can be obtained and, overall, the way in which this information can be combined with that from other sources to penetrate the complexity of membrane protein structure and function. We hope that new researchers will find the book of help in assessing current methodology whilst

more experienced practitioners may benefit by extending their knowledge of areas which lie outside their particular expertise. We would also hope that the book will convey to all its readers something of the fascination which membrane proteins exert over us.

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1

Analysis of membrane protein composition by gel electrophoresis

C. IAN RAGAN

1.1 Introduction

Electrophoretic separation of proteins in gels is a universally popular technique whose application is not, of course, restricted to membrane proteins. Nevertheless, the need in membrane protein research for techniques for the separation and analysis of complex mixtures of hydrophobic proteins has perhaps provided a major impetus for the development of improved electrophoretic methods over the last decade, particularly the separation of sodium dodecyl sulphate (SDS)–protein complexes on polyacrylamide gels. The latter technique, and the complementary use of isoelectric focusing methods (Chapter 2), are the basis of many aspects of membrane protein research. In view of this importance, I make no apology that this chapter describes electrophoretic methods which can equally be applied to soluble proteins. However, I have concentrated on those methods which have proven to be most useful for membrane proteins and highlighted particular problems which may be encountered in membrane research.

In principle, electrophoretic separation of proteins can be carried out under conditions either where the native conformation of the protein is preserved or where the protein is denatured. For membrane proteins, solubilization with preservation of structure is generally achieved by the use of mild detergents. The protein is then in a detergent or detergent/lipid micelle and can be separated from other such proteins by electrophoresis. As with many other

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protein separation techniques, the resolution that can be achieved is less than that found with water-soluble proteins, and the method is of limited utility. Superior resolution can be achieved by electrophoresis under denaturing conditions, of which the most common denaturant is SDS. The latter is therefore the most widely used method for analysis of membrane proteins and, as a reflection of this, the bulk of this chapter is concerned with electrophoresis of SDS–protein complexes in polyacrylamide gels. Other less commonly used denaturants and electrophoresis in non-denaturing gels are given briefer coverage at the end of the chapter.

1.2 Gel electrophoresis in SDS

The interaction of a large number of soluble proteins with SDS has been extensively studied by Tanford, Reynolds and co-workers (Reynolds and Tanford, 1970; Fish *et al.*, 1970; Makino *et al.*, 1974). Most of these proteins were found to bind close to 1.4 g of SDS per g of protein, or one molecule of SDS per two amino acid residues (Nielson and Reynolds, 1978) and adopted a unique conformation in which the Stokes radius was a function of the molecular weight only. This property has been exploited to provide a simple and powerful method for the separation of polypeptides and measurement of their molecular weights by electrophoresis of SDS–protein complexes through polyacrylamide gels of various porosities. The fact that some proteins do not bind the usual amount of SDS or do not migrate in electrophoresis strictly according to their molecular weights does not, of course, prevent them from being separated from other proteins by this method. Therefore, I have considered separation methods and molecular weight determination as separate issues.

1.2.1 SOLUBILIZATION OF MEMBRANE PROTEINS BY SDS

The ability of SDS to solubilize otherwise recalcitrant membrane proteins accounts for the popularity of SDS-gel electrophoresis. The original conditions of Weber and Osborn (1969) are still widely used (viz. 1% SDS, 1% 2-mercaptoethanol, up to 1 mg of protein per ml in 0.01 M sodium phosphate, pH 7.0, 100° C for 2 min) and appear to be suitable for the majority of proteins. The presence of 2-mercaptoethanol to reduce disulphide bonds is necessary for molecular weight measurements since it enables polypeptide chains to unfold completely. However, it is not obligatory and treatment with, say, *N*-ethylmaleimide and SDS may be desirable where interchain disulphide bonds need to be preserved (for example after cross-linking with a disulphide-containing bifunctional reagent; Wang and Richards, 1974). Weber *et al.* (1972) discussed various problems that may arise with the standard solubilization procedure. Firstly, heating may give rise to limited hydrolysis of

the polypeptide. Omitting the 100° C step may solve this but may then render the sample prone to proteolytic digestion from endogenous proteases, particularly in crude preparations. Addition of protease inhibitors is then advisable (e.g. phenylmethanesulphonyl fluoride for serine proteases, *o*-phenanthroline for divalent cation-dependent proteases and *N*-ethylmaleimide for cysteine proteases). Alternative solubilization procedures such as 7–8 M guanidine hydrochloride and 2-mercaptoethanol followed by alkylation of sulphydryl groups with iodoacetamide may be used (Weber *et al.*, 1972) but these conditions are unlikely to solubilize all membrane proteins. An additional problem with membrane proteins is that of aggregation. Proteins containing a high proportion of hydrophobic amino acids may aggregate with themselves or other such proteins, even in the presence of SDS, and fail to enter the gel. Diverse conditions may promote this: high temperature, organic solvent extraction, trichloroacetic acid precipitation, and freeze–thawing (Briggs and Capaldi, 1977; Merle and Kadenbach, 1980). Aggregates are usually visible as a band on top of the gel or even in the stacking gel (when used). To obviate this problem, solubilization at ambient temperature (perhaps aided by sonication) and the presence of additional denaturants such as urea (Ludwig *et al.*, 1979) should be tried. There are no rules, but it is advisable to try several solubilization strategies with a new membrane system.

1.2.2 COMPOSITION OF POLYACRYLAMIDE GELS AND BUFFERS

An enormous variety of gel configurations and gel or buffer compositions is available for separation of SDS–protein complexes. A good starting point is the original procedure of Weber and Osborn (1969) which employed gels containing a fixed single concentration of acrylamide (e.g. 10% w/v) and bisacrylamide (1:37 by weight) as the cross-linker. A continuous buffer system of sodium phosphate containing SDS was used and electrophoresis was carried out on gels cast into cylindrical tubing. In the search for improved resolution and convenience, there have been developments in three areas: the shape of the gel, the buffer systems used and the composition of the gel.

(a) *Slab gels and cylindrical gels*

Cylindrical gels have now been largely superseded by slab gels. Under otherwise identical conditions, resolution on typical thin slab gels (e.g. 1.5 mm thick) is better than in typical cylindrical gels (e.g. 5 mm thick). An important factor is more even temperature control, particularly in those types of apparatus in which the slab gel is totally immersed in circulating buffer. A common problem with air-cooled gels is 'smiling' of the protein bands caused by overheating of the middle of the gel. This, of course, is worse the thicker the gel layer. The thinness of slab gels conveys other advantages. They can be

more easily dried down for radioautography or fluorography (Section 1.2.3.c); it is easier to infiltrate other reagents for protein staining (Sections 1.2.3.a and 1.2.3.b) or immunological identification (Section 1.2.3.d) and protein bands can be transferred on to nitrocellulose paper or other transfer media for further analysis (Section 1.2.5). Molecular weight determination (Section 1.2.4) is facilitated by running several samples in parallel tracks on the same slab gel. Mobility between tracks is more reproducible than between apparently identical cylindrical gels. Finally, complex gel compositions such as gradients of acrylamide can be conveniently cast in slab form but not in cylindrical form.

The only disadvantage of thin slab gels is their lower sample capacity. For example, for analysis of radioactive proteins of low specific radioactivity, and when resolution is not paramount, cylindrical gels may be more convenient than thick slab gels and are more easily sliced and counted (Sections 1.2.3.c).

Several commercial designs for slab gel electrophoresis are available, but many workers prefer to build their own. A convenient design for simultaneous casting of several slab gels is described below (Section 1.2.2.c).

(b) *Continuous and discontinuous buffers*

Despite their added complexity, discontinuous buffer systems are now largely used in preference to continuous systems. The gel consists of two parts: a short (1–2 cm) stacking gel of large porosity and a separating gel. Different buffer compositions are used for the two gels and in the electrode compartments. The theory of such 'disc' systems has been described by Ornstein (1964) and Davis (1964) and they have been widely adopted for separation of SDS–protein complexes by the simple addition of SDS to some or all of the buffers. The samples are applied to the stacking gel in which they are concentrated to a very small volume ('stacking'). The change in buffer composition when the separating gel is reached causes 'unstacking' and the SDS–protein complexes separate as in continuous systems. The initial concentration to a small volume results in improved resolution and enables larger sample volumes to be handled with no loss of resolution. Several buffer systems have been described of which the most popular is that of Laemmli (1970). Others have been devised by Jovin *et al.* (1971) and the use of two of these is fully discussed by Neville and Glossman (1974). From my own experience they all produce very similar results. In Table 1.1, the compositions and properties of these systems are given. The drawback of disc systems is that they only work properly with scrupulous attention to the buffer composition and pH. In particular, high-ionic-strength samples do not stack properly and dilute protein samples may have to be dialysed, for example, to get them into an appropriate buffer. In gels of constant composition containing relatively low acrylamide (e.g. 10–12%), low-molecular-weight polypeptides may not unstack from the dye front. A brief inspection of published work reveals how common this is, but it is rarely referred to. The problem again lies in the buffers; particularly if these are not

Table 1.1 Composition of some discontinuous buffer systems for SDS-gels
The systems J4179 and J3561 of Jovin *et al.* (1971) are described by Neville and Glossmann (1974). These have different buffers for the separating gel, the running gel and the two electrode compartments. The Laemmli (1970) system employs the same buffer for both electrode compartments.

Buffer	*J4179*	*J3561*	*Laemmli*
Separating gel	0.427 M Tris–HCl, pH 9.18	0.0716 M imidazole–HCl, pH 4.67	0.375 M Tris–HCl, 0.1% SDS, pH 8.8
Stacking gel	0.0541 M Tris–H_2SO_4, pH 6.14	0.0694 M imidazole–H_2SO_4, pH 4.72	0.125 M Tris–HCl 0.1% SDS, pH 6.8
Cathode	0.0406 M Tris–boric acid, 0.1% SDS, pH 8.64	0.0362 M Tris–imidazole, 0.1% SDS, pH 8.0	0.25 M Tris, 0.192 M glycine, 0.1% SDS
Anode	0.427 M Tris–HCl, pH 9.5	0.733 M imidazole, 0.136 M HCl	

freshly prepared. The easiest way out is to decrease the porosity of the gel so that small proteins do not migrate close to the dye front.

(c) *Composition of gels*

Polyacrylamide is a linear polymer and must be cross-linked to provide pores for separation of SDS–protein complexes. Bisacrylamide is certainly the most common cross-linker and is generally used at a concentration of one part to approximately 30 parts of acrylamide (Weber and Osborn, 1969). Other cross-linkers are described in Section 1.2.3c. Variation of the pore size of gels can be achieved by varying the amount of the acrylamide–bisacrylamide mixture (expressed usually just as the percentage by weight of acrylamide), the acrylamide to bisacrylamide ratio, or by addition of other reagents to the mixture such as urea or glycerol.

Gels which cover the widest range of polypeptide sizes are those which have a gradient of acrylamide increasing from the origin to the end of the gel. Linear or exponential gradients can be used and the concentrations and shape of the gradient are entirely a matter of choice. The effect on protein bands of moving always into a region of smaller porosity is to prevent them from spreading by diffusion and greatly increases the resolution, particularly of smaller proteins. With gradients covering a wide variation in polyacrylamide concentration, the proteins may eventually become almost stationary, but this is not necessary for a considerable enhancement in the resolution. Used with discontinuous buffer systems, gradient gels have the additional advantage that small proteins no longer fail occasionally to unstack since they inevitably encounter an acrylamide concentration where they leave the ion front. These gels are therefore more tolerant of ageing or imprecisely made up buffers.

Single-gradient slab gels can be made as described by Douglas *et al.* (1979) using a gradient mixer to deliver the monomer solutions. Gels containing 10% to 15% acrylamide separate proteins in the range 150 to 10 kDa. The acrylamide concentrations can be varied at will, but some adjustment to polymerizing reagent concentrations may also be necessary. It is advisable to ensure that the gel polymerizes from the top downwards (lower to higher concentration) to prevent convection currents disturbing the gradient. For routine use of gradient gels it is an advantage to be able to cast several at one time. This can be done using the apparatus shown in Fig. 1.1. Cassettes of the desired dimensions are constructed from glass plates, Perspex spacers and gel sealing tape of the type supplied by several manufacturers of electrophoretic equipment. Baking the assembled cassettes at 100° C overnight prevents the tape from lifting off during storage of the gels in buffer. The cassettes are stacked in the apparatus which is first filled with 5% (v/v) aqueous ethanol to act as an overlay. The monomer is then admitted from underneath via a gradient mixer and finally sucrose solution is admitted to push the acrylamide up to within 1–2 cm of the top of the cassettes. After setting, the solid block of

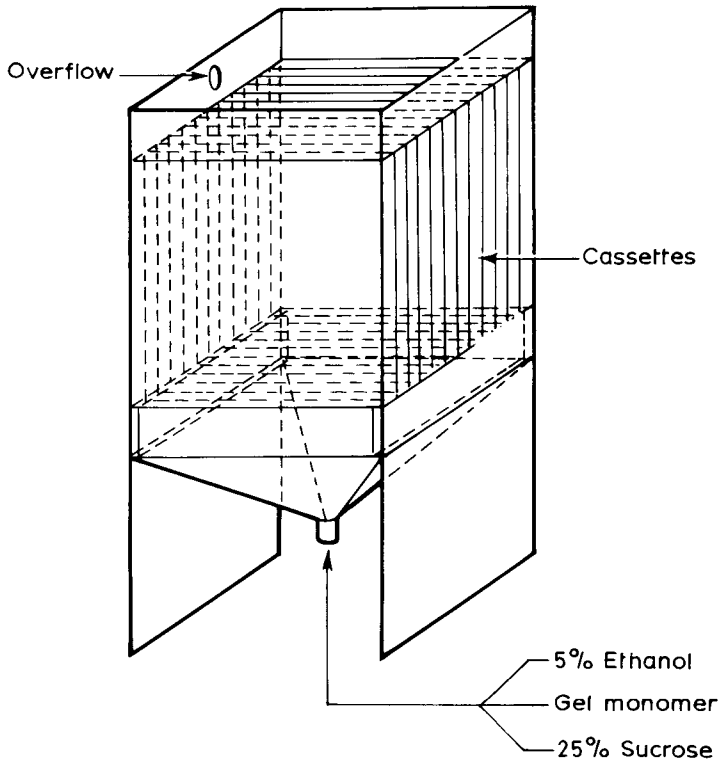

Fig. 1.1 Apparatus for simultaneous casting of several slab gels. The apparatus is constructed out of Perspex. After filling with assembled cassettes, 5% (v/v) ethanol is admitted through the bottom. This is displaced through the overflow by acrylamide monomer from a gradient mixer followed by sucrose underlay until the acrylamide is approximately 1 cm from the top of the cassette. This space is used for a stacking gel and sample comb. Apparatus of this type is available from Universal Scientific Ltd., London, UK.

cassettes is extruded by water pressure, excess acrylamide is scraped off and the gels are stored in running gel buffer in the cold. The method of casting removes the need to use grease or clamps to seal the cassettes and spacers together. Immediately before use, a stacking gel is cast on the top of the running gel with slots for samples. These gels can be kept for weeks without any alteration in running characteristics.

While gradient gels may provide adequate resolution of small proteins (2–10 kDa), there are other methods. The first of these was that of Swank and Munkres (1971) who introduced a still popular modification of the original Weber and Osborn (1969) procedure which was to include 7–8 M urea in the sample and gel and increase the acrylamide concentration and bisacrylamide to acrylamide ratio. The urea reduces the pore size giving better separation of