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Internal localization of *Micrococcus lysodeikticus* membrane ATPase by iodination with ^{125}I

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SUMMARY

Micrococcus lysodeikticus membrane ATPase is inaccessible to ^{125}I labeling when stabilized protoplasts are reacted with the lactoperoxidase–$Na^{125}I$ system or with ^{125}ICl. Membranes isolated from protoplasts or by the standard procedures used in this laboratory when labeled with ^{125}I yield a major labeled peak in sodium dodecyl sulfate–polyacrylamide gels which is identifiable as ATPase subunit protein. The results are in accord with the location of the ATPase on the inner face of the membrane. Lipid extracted by the Bligh and Dyer (Bligh, E.G. and Dyer, W.J. (1959) *Can. J. Biochem. Physiol.* 37, 911–917) method is also labeled by the iodination procedures.

The asymmetric distribution of molecules detectable on the outer and inner faces of biomembranes has been established in recent years by a variety of chemical and enzymatic labeling procedures[1–7] and by determining the sensitivity of external components to enzymatic digestion[8]. Further knowledge of the external–internal arrangement of molecules in cell membranes has thus been gained from covalent labeling of exposed reactive groups of proteins, glycoproteins and lipids in intact cells and isolated membranes by either chemical or enzymatic labeling procedures[1–7]. One of the simpler techniques involving ^{125}I iodination of proteins by lactoperoxidase[9], has now been applied to a number of cell surface membrane studies and the surface location of certain proteins and glycoproteins has been reported[2,3,7,10,11]. Considerable success has thus been achieved in studying the disposition of membrane components by a combination of labeling and identification of labeled components by sodium dodecyl sulfate–polyacrylamide gel electrophoresis[2,3,7,11].

Previous studies in our laboratory have shown that the antibody-combining sites of

the *Micrococcus lysodeikticus* membrane Ca^{2+}-activated ATPase are not accessible on the outer surface of the protoplast membrane, thus suggesting an internal localization of this membrane enzyme[12]. It therefore appeared conceivable that the iodination procedure would yield further evidence on the asymmetric distribution of this enzyme on the inner or outer surface of the membrane and this paper presents the results of our study.

M. lysodeikticus (NCTC 2665) was grown, harvested and membranes isolated as described in previous publications from this laboratory[13,14]. The membrane Ca^{2+}-activated ATPase was purified as previously reported[15] and appeared to be homogeneous by several criteria (single band in polyacrylamide gel, a single line of precipitate in double-diffusion agar gel and immunoelectrophoresis tests against specific antiserum, uniformity of particles seen in the electron microscope)[15–17]. Stabilized protoplasts were prepared by lysozyme treatment of cells suspended in 0.05 M Tris–HCl buffer, pH 7.5, containing 0.8 M sucrose and 25 mM $MgCl_2$. Wall digestion and protoplast formation was allowed to proceed for 30–40 min at about 24 °C and the protoplasts were washed twice on the centrifuge (9000 × *g*, 30 min) with the Tris–sucrose–Mg^{2+} stabilizing medium which was also used for final resuspension of the protoplasts. The membrane fraction from stabilized protoplasts was also prepared for iodination, by lysing the protoplasts in 0.05 M Tris–HCl, pH 7.5, and recovering the membranes and washing by the standard procedures used in this laboratory[13,14].

Two iodination procedures were used. The lactoperoxidase method[3,10] using $Na^{125}I$ from New England Nuclear and lactoperoxidase purchased from Nutritional Biochemicals, was performed at room temperature (22–24 °C) for 15 min at which time the preparations were brought to 0 °C on an ice bath for further processing. The reaction mixtures, consisting of either membranes or protoplasts suspended in 0.05 M Tris, pH 7.5, and 0.05 M Tris–0.8 M sucrose–25 mM $MgCl_2$, respectively, contained 200–400 μCi $Na^{125}I$ (carrier free, neutralized to pH 7.0 with NaOH), 300 μg lactoperoxidase and 4.5 mM H_2O_2 in 1.0 ml. Radioiodination of proteins can also be achieved with a number of reactive agents but according to Roholt and Pressman[18] ICl is the reagent choice. The availability of high specific activity ^{125}ICl (New England Nuclear) has led to its use in iodinating proteins in several laboratories (Schwartz, J.H., personal communication) and it has accordingly been employed in the present investigation. To iodinate the protoplasts (in Tris–sucrose–Mg^{2+}), membrane fractions and ATPase (in 0.05 M Tris–HCl, pH 7.5), 1-ml volumes of the suspensions or solutions were layered on 50 μl of the ^{125}ICl (200 μCi) dissolved in carbon tetrachloride (spec. act. 100 Ci/mole, New England Nuclear) at 0 °C and the two phases mixed by gentle, periodic shaking and allowed to react for 20 min at 0 °C. The aqueous phases were carefully removed from the lower organic solvent phase with a chilled Pasteur pipette and placed in 12-ml heavy-wall, Pyrex glass centrifuge tubes at 0 °C for further processing.

Iodinated protoplasts were washed twice with the Tris–sucrose–Mg^{2+} medium on the centrifuge, then lysed in 0.05 M Tris–HCl and the membranes recovered from the labeled protoplasts by the usual procedures[13,14]. The iodinated membranes were removed from the reaction mixtures (lactoperoxidase–$Na^{125}I$, or ^{125}ICl–buffer phases) by

centrifugation and washing a minimum of three times with 0.05 M Tris–HCl as previously described[14]. After iodination, the ATPase was transferred to dialysis tubing and dialysed against several changes of 0.05 M Tris–HCl, pH 7.5. In some experiments the ATPase was precipitated with specific antiserum[16] (generously supplied by Dr Joel Oppenheim) and the specific precipitates washed with buffered saline prior to further examination.

Membrane components exposed on outer and inner faces of the membrane which possess groups susceptible to iodination have been detected by examination of the labeling patterns[2,3,7,10,11] seen on dissociation of the membranes and electrophoresis by the sodium dodecyl sulfate–polyacrylamide gel method of Weber and Osborn[19]. We have accordingly determined the patterns of "subunit" labeling of membranes from iodinated protoplasts, iodinated isolated membranes and iodinated purified ATPase and its specific precipitates with antisera. Fractions were dissolved in and dialysed against two changes of 2% sodium dodecyl sulfate–2% mercaptoethanol[19] for 16 h at 37 °C and in some instances the preparations were heated for 3 min at 100 °C before electrophoresis. Since it has been known from the work of Salton and Schmitt[20] that lipids dissolved in detergents migrate in polyacrylamide gels and in view of the suggestions that some of the fast-moving components in sodium dodecyl sulfate–polyacrylamide gels may be of lipid or glycolipid nature[8,21], we have also examined iodinated membranes which had been subjected to the Bligh and Dyer[22] extraction procedure. The lipid fraction was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis[19], in parallel with the extracted, insoluble membrane protein residue which had to be heated in the 2% sodium dodecyl sulfate–2% mercaptoethanol for dissolution.

Membranes from protoplasts iodinated by either the lactoperoxidase method or reaction with ^{125}ICl showed very little labeling and no distinctive patterns were obtained in the sodium dodecyl sulfate–polyacrylamide gels (Fig. 1). It would thus appear that relatively few residues capable of being iodinated are freely exposed to the outer membrane surface. However, the results of iodinating the isolated membranes were in marked contrast as seen in the sodium dodecyl sulfate–polyacrylamide pattern obtained with lactoperoxidase labeling (Fig. 1). Similar distinctive patterns of "subunit" labeling were also seen with isolated membranes reacted with ^{125}ICl. The more extensive labeling of the isolated membranes in this bacterial system is thus in good agreement with the results recently reported for mammalian cell membranes[7]. It is also apparent that significant labeling of the membrane lipid occurs as illustrated in the pattern obtained with iodinated membranes which had been extracted by the Bligh and Dyer[22] method. The residual membrane protein fraction after extraction (Fig. 2) was electrophoresed in parallel with the extracted membrane lipids (Fig. 3) and the results clearly indicate that one of the major peaks in the sodium dodecyl sulfate–polyacrylamide gel patterns can be accounted for by the labeled lipid. These patterns of labeling (Figs 1–3) are contrasted with that obtained with ATPase iodinated with ^{125}ICl (Fig. 4). The labeling of ATPase by the lactoperoxidase method also gave similar results.

Thus one of the major ^{125}I-labeled peaks of iodinated membranes (Figs 1 and 2) corresponds to that obtained with purified ATPase (Fig. 4) and is identifiable as the ATPase

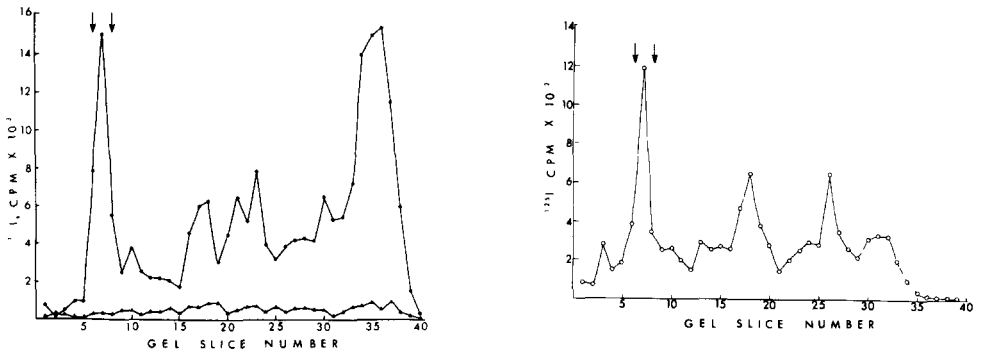

Fig. 1. Pattern of distribution of ^{125}I radioactivity in *M. lysodeikticus* membrane fractions iodinated with lactoperoxidase and electrophoresed in sodium dodecyl sulfate–polyacrylamide gels by the method of Weber and Osborn[19]. ▲——▲, the pattern obtained with membranes isolated from iodinated protoplasts; and ●——●, the results obtained with isolated membranes subjected to the iodination procedure. Initial reaction mixtures of membrane or protoplast suspensions contained approx. 2–4 mg membrane protein (stabilized protoplast suspension contained about 4 times this amount as total protein to give the above equivalent membrane protein concentration) 200–400 μCi $Na^{125}I$, 300 μg lactoperoxidase, 4.5 mM H_2O_2 per sample tube of 1 ml (0.05 M Tris for membranes; 0.05 M Tris, 0.8 M sucrose, 25 mM Mg^{2+} for protoplasts). Membranes were recovered after the standard washing in 0.05 M Tris–HCl buffer, pH 7.5, and were dissolved in 2% sodium dodecyl sulfate–2% mercaptoethanol and dialysed as described in the text. Samples containing approx. 100–200 μg protein were electrophoresed. The region where the ATPase subunits band in the gel is shown by the arrows.

Fig. 2. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis pattern of residual membrane protein fraction obtained after extraction of the lipids (Bligh and Dyer[22] method) from isolated membranes labeled with ^{125}I by the lactoperoxidase method. Labeling conditions were as for Fig. 1. The amount of protein per gel was about one-tenth of that used for Fig. 1. ATPase subunit region is indicated by the arrows.

subunit region (mol. wts 62 000 and 60 000) in sodium dodecyl sulfate–polyacrylamide gels as shown recently by Salton and Schor[23]. No significant labeling of these components occurred when protoplasts were iodinated (Fig. 1) and this result does not appear to be due to a complete inhibition of the iodination reactions by the protoplast stabilizing medium (membranes iodinated in this medium gave gel patterns similar to those obtained in Tris buffer). The identity of the labeled peak region with ATPase subunits[23] was further substantiated by subjecting the iodinated membranes to the shock-wash which selectively releases ATPase from the membranes[15]. A marked decrease in the major ATPase peak (Fig. 1) occurred and labeled material giving rise to a peak identical to that in Fig. 4 was recovered from the shock-wash by specific precipitation with anti-ATPase and electrophoresis of the precipitates (dissolved in 2% sodium dodecyl sulfate–2% mercaptoethanol) in the Weber and Osborn system[19].

These results have thus established that the ATPase membrane protein is inaccessible to labeling when the outer membrane surface of protoplasts is subjected to the two iodination procedures. However, when membranes are prepared from lysed protoplasts or by the direct lysis of cells by our standard procedures[13,14], the ATPase protein can be

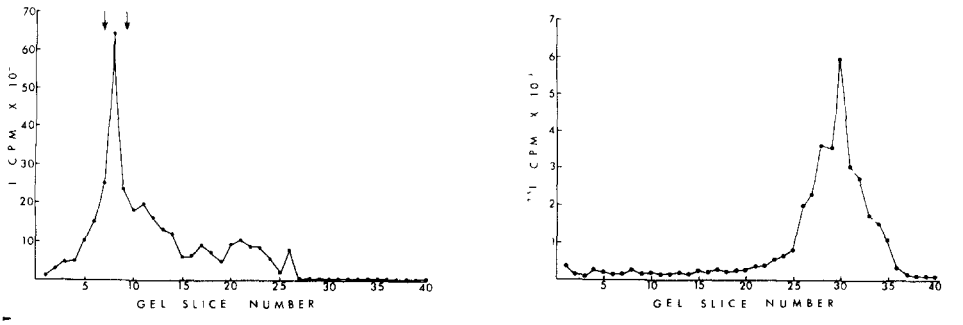

Fig. 3. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis pattern of the lipid extracted from the iodinated isolated membranes (conditions as for Fig. 1) by the Bligh and Dyer[22] procedure. The total lipid extract was dissolved in 2% sodium dodecyl sulfate–2% mercaptoethanol for electrophoresis.

Fig. 4. The sodium dodecyl sulfate–polyacrylamide gel electrophoresis pattern obtained with purified ATPase iodinated with ^{125}ICl. Purified ATPase (150 μg protein per ml 0.05 M Tris–HCl, pH 7.5) was iodinated by layering 1 ml solution on 50 μl ^{125}ICl (200 μCi, spec. act. 100 Ci/mole) in carbon tetrachloride as described in the text, the aqueous phase removed and dialysed for 24 h against several changes of 0.05 M Tris and finally dialysed for 12 h at 37 °C against three changes of 2% sodium dodecyl sulfate–2% mercaptoethanol for electrophoresis by the method of Weber and Osborn[19]. Approx. 10 μg protein was electrophoresed. The two ATPase subunits band in the region indicated by the arrows and the additional minor labeled components are probably attributable to the associated proteins of the shock-wash ATPase as reported by Salton and Schor[23]

identified as a major iodinated product. These results are therefore in accord with the suggestions that the ATPase is located on the inner face of the membrane and have thus extended the iodine labeling method to establishing the asymmetric distribution of a membrane enzyme.

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