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ANALYSIS OF *STAPHYLOCOCCUS AUREUS* CYTOPLASMIC MEMBRANE PROTEINS BY ISOELECTRIC FOCUSING

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Cytoplasmic membranes were isolated from late-exponential phase *Staphylococcus aureus* 6538 P and the membrane proteins examined under non-denaturing conditions by thin-layer isoelectric focusing (TLIEF) in a pH 3.5–9.5 gradient. Isolated membrane preparations retained protein integrity as judged by the demonstration of membrane bound adenosine triphosphatase (ATPase) activity in addition to four other solubilized membrane enzyme markers. Membranes were effectively solubilized with 2.5% Triton X-100 (final concentration). Examination of Triton X-100 solubilized membrane preparations established the presence of 22 membrane proteins with isoelectric points between 3.7 and 6.0. The focused proteins displayed the following enzymatic activities and isoelectric points by zymogram methods: ATPase (EC 3.6.1.3), 4.20; malate dehydrogenase (EC 1.1.1.37), 3.90; lactate dehydrogenase (EC 1.1.1.27), 3.85; two membrane proteins exhibited multiple bands upon enzymatic staining: NADH dehydrogenase (EC 1.6.99.3), 4.25, 4.35; succinate dehydrogenase (EC 1.3.99.1), 4.85, 5.10, 5.35.

Introduction

Studies on the cytoplasmic membrane proteins from *Staphylococcus aureus* require the removal of the cell wall and the separation and purification of the cytoplasmic membrane from intracellular material. The inability to adequately remove the cell wall from this bacterium has resulted in a limited survey of staphylococcal membrane proteins. However, documentation for the isolation of homogeneous membranes from *S. aureus* 6538P has been presented [1,2]. The production of lysostaphin-induced *S. aureus* protoplasts [3] assures the removal of cell wall material facilitating membrane isolation. Membranes prepared by this procedure appear as transparent vesicles by cytological examination and contain a characteristic membrane biochemical composition [2]. As our interests are directed towards an understanding of the involvement of these proteins in membrane physiology, the use of *S. aureus* 6538P affords a reliable system for the analysis of staphylococcal membrane proteins. Using isolated membranes we previously reported [2] on some of the unique features of one staphylococcal membrane protein, the adenosine triphosphatase (ATPase). Therefore, because of the paucity of data regarding staphylococcal membrane protein composition, this investigation addresses the basic problem of the separation and identification of *S. aureus* membrane proteins under non-denaturing conditions.

The results presented establish the protein composition and isoelectric point profile of isolated *S. aureus* 6538P cytoplasmic membrane proteins in addition to identifying several membrane enzymes and isoenzymes by zymogram methods.

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Abbreviations: TLIEF, thin-layer isoelectric focusing; TNBT, tetranitrobluetetrazolium; PMS, phenazine methosulfate; PMSF, phenylmethanesulfonyl fluoride.

Methods

Membrane isolation. Cytoplasmic membranes were prepared from late exponential phase *Staphylococcus aureus* ATCC 6538P as previously described [2]. Briefly, washed cells were resuspended in the protoplasting mixture (5.5 g wet weight/20 ml) containing hypertonic buffer (50 mM Tris-HCl, pH 7.5, 15 mM $MgSO_4$, 3.45 M NaCl) and treated with lysostaphin (21 μg/ml) and DNAase (25 μg/ml) for 65 min. The protoplasts were removed by centrifugation at 10000 $\times g$ for 30 min and subsequently lysed by resuspending in hypotonic buffer (50 mM Tris-HCl, pH 7.5, 15 mM $MgSO_4$) containing RNAase (50 μg/ml) and DNAase (50 μg/ml). The lysate was centrifuged at low speed (2000 $\times g$) for 10 min. The supernatant was centrifuged again at low speed to remove any unlysed cells and then at 35000 $\times g$ for 25 min to obtain the membrane fraction. This fraction was washed once in hypertonic buffer to remove any ionically-associated cytoplasmic material and then five times in hypertonic buffer at 35000 $\times g$ for 25 min.

The membranes were used immediately for enzymatic and electrophoretic analyses or stored in 1 ml suspensions (5.5 mg protein/ml) at −70°C in the presence of, by weight, 1 part phenylmethylsulfonyl fluoride (PMSF) to 20 parts membrane protein.

Membrane solubilization by Triton X-100 and thin-layer isoelectric focusing (TLIEF). Isolated membrane preparations (5.5. mg protein/ml) were solubilized in 10 mM Tris-HCl, pH 7.5, containing 2.5% (v/v, final concentration) Triton X-100 for 30 min at 22°C. The mixture was centrifuged at 35000 $\times g$ for 30 min at 4°C and the supernatant retained for analysis. The supernatant contained in excess of 90% of the membrane protein.

TLIEF was performed as described by Winter et al. [4] with minor modifications; the thin-layer gel contained 10 ml 20.1% (w/v) acrylamide, 10 ml 0.9% (w/v) bis, 7 ml 87% (v/v) glycerol, 3.6. ml ampholyte solution, 0.6 ml 10% (v/v) Triton X-100, and 30 ml distilled water. The ampholyte solution included the following pH ranges: 2.8 ml of pH 3.5–10, 0.4 ml of pH 9–11, 0.2 ml of pH 5–7, and 0.2 ml pH 4–6. The solution was stirred for 10 min under reduced pressure and 1.5 ml 1% (w/v) ammonium persulfate was added. The mixture was injected into a mold (260 × 115 mm) separated by a 2 mm rubber gasket secured with clamps and polymerized for 1 h.

Solubilized membrane proteins were placed on rectangles (6 × 12 mm) of Whatman No. 3 filter paper, or in plastic frames (6 × 12 × 2 mm) for dilute protein extracts. Routinely, 140 μg of solubilized membrane protein was focused. The samples were placed near the cathode after determining a suitable application site between electrodes. An electrode strip was soaked in the respective electrode solution (anode: 1 M H_3PO_4; cathode: 1 N NaOH), placed at the proper gel edge, and focusing was performed in an electrofocusing apparatus (LKB, model 2117) on a 4°C cooling plate at a constant current of 50 mA for 30 min; the mode was then switched to maintain a current of 40 mA by regularly increasing the voltage to 1000 V. Electrofocusing was completed when the current fell to 25 mA at 1000 V in a total time of 90 min. For protein staining, focused gels were fixed in 3.5% (w/v) sulfosalicylic acid, 11.5% (w/v) trichloroacetic acid for 1 h, rinsed in destaining solution (25% (w/v) ethanol, 8% (v/v) acetic acid), and then stained with 0.12% (w/v) Coomassie brilliant blue R-250 dissolved in destaining solution for 30 min at 60°C.

The pH gradient was determined from a lane of focused gel by slicing the gel into 5 mm sections, placing the slice into 1 ml of previously boiled distilled water, and recording the pH after 1 h shaking at 22°C.

Zymogram analysis in TLIEF. Enzymatic activities of the focused membrane proteins were determined as described originally by Wadstrom and Smyth [5]. Zymograms for ATPase, NADH dehydrogenase, malate dehydrogenase, and succinate dehydrogenase were performed as described by Owen and Salton [6] modified in this study in order to accomodate a thin-layer isoelectric focusing gel.

Following TLIEF, the lane(s) of the gel containing the focused membrane proteins was cut from the slab and incubated in the respective mixture at 37°C in a closed humid chamber for 1 h. (a) ATPase (EC 3.6.1.3): the gel was rinsed for 5 min in 0.4 M Tris-acetate (pH 7.0) and then placed in a solution containing 20 ml 20 mM ATP (pH 7.0), 3 ml 2% (w/v) lead acetate, 5 ml 0.1 M $MgSO_4$, 20 ml 0.1 M Tris-acetate and 2 ml distilled water. Following incubation, the gel was rinsed with distilled water and developed with 0.1% (w/v) Na_2S. (b) NADH dehydro-

genase (EC 1.6.99.3): the gel was rinsed for 5 min in 0.4 M Tris-HCl (pH 7.5) and then placed in a solution containing 7 mg NADH, 4 mg tetranitrobluetetrazolium (TNBT), 20 ml 0.1 M Tris-HCl (pH 7.5), and 10 ml 1.5% (w/v) buffered agarose. (c) Succinate dehydrogenase (EC 1.3.99.1): the gel was rinsed for 5 min in 0.4 M Tris-HCl (pH 7.5) and then placed in a solution containing 2 ml 1 M disodium succinate, 1 ml 0.1 M KCN, 6 mg TNBT, 17 ml 50 mM Tris-HCl (pH 7.5), and 10 ml 1.5% buffered agarose. (d) Malate dehydrogenase (EC 1.1.1.37): the gel was rinsed for 5 min in 0.1 M Tris-HCl (pH 7.5) and then placed in a solution containing 2 ml 1 M sodium DL-malate, 10 mg NAD, 4 mg TNBT, 0.5 mg phenazine methosulfate (PMS), 1 ml 0.1 M KCN, 17 ml 50 mM Tris-HCl (pH 7.5), and 10 ml 1.5% (w/v) buffered agarose. (e) Lactate dehydrogenase (EC 1.1.1.27) was detected by the method of Van der Helm [7]. The gel was rinsed for 5 min in 0.4 Tris-HCl (pH 7.4) and then placed in a solution containing 4 ml 1 M sodium DL-lactate, 40 mg NAD, 4 ml 40 mM $MgCl_2$, 4 ml 0.1 M KCN, 5 mg TNBT, 1 mg PMS, 10 ml 50 mM Tris-HCl (pH 7.4), and 10 ml 1.5% (w/v) buffered agarose. (f) Alkaline phosphatase (EC 3.1.3.1) was detected by the method of Smith et al. [8]. The gel was rinsed for 30 min in 16 mM boric acid, 50 mM KOH (pH 9.7) and then placed in a solution containing 6 mg 4-methylumbelliferone phosphate, 18 ml borate-KOH buffer with 10 mM $MgCl_2$, and 9 ml 0.7 mM $ZnSO_4$. Following incubation for 2 h at room temperature, the gel was examined under ultraviolet light ar 360 nm.

Analytical procedures. Staphylococcal membrane ATPase activity was measured by the liberation of inorganic phosphate as previously described [2]; the standard incubation mixture contained 100 mM KCl, 4 mM $MgCl_2$, 2.5 mM ATP and 50 mM Tris-acetate buffer (pH 6.5).

Protein was assayed by the method of Lowry et al. [9] modified according to Wang and Smith [10] to facilitate determinations in the presence of detergents. Bovine serum albumin was used as the standard in this analysis.

Results

Membrane enzyme acitivity during isolation.

As the bacterial adenosine triphosphatase (ATPase) is most commonly a membrane-bound enzyme [11–13] the distribution of this membrane marker enzyme among various fractions throughout the membrane isolation procedure was examined. The monitoring of membrane ATPase activity during membrane isolation allows for a determination of the effects of cellular fractionation and enzymatic treatment on membrane-enzyme integrity. Of the 5 fractions examined (protoplast supernatant, whole cell lysate, low speed pellet, cytoplasmic content, and membrane fraction), the ATPase activity was located predominantly in the membrane fraction (Table I). The specific activity of the associated ATPase was approximately one hundred times greater than that found in the cytoplasm and about 4-times greater than that found in the low speed pellet. It was not surprising to find activity in the low speed pellet as this fraction contained some membranes still associated with some unlysed cells that were sedimentable under these conditions.

Solubilization of isolated S. aureus membranes and analysis by TLIEF

To assess the protein heterogeneity, isoelectric points, and biological activities of the membrane proteins under non-denaturing conditions, *S. aureus* membranes were examined by TLIEF. Prior to isoelectric focusing, the membrane preparations were treated with triton X-100. A marked improvement in membrane protein extraction was observed when less con-

TABLE I

ATPase ACTIVITY DURING MEMBRANE ISOLATION FROM *S. AUREUS* 6538P

Specific activity = μmol P_i released per 30 min per mg protein. Data represent the mean values from six separate experiments carried out in duplicate ± S.D. The reaction mixture contained: 2.5 mM ATP, 100 mM KCl, 4.0 mM $MgCl_2$, 50 mM Tris-acetate (pH 6.5), and 150 μg protein of the fraction to be tested for activity.

Fraction	Specific activity
Protoplast supernatant	0.03 ± 0.01
Whole cell lysate	0.70 ± 0.20
Low speed pellet	1.07 ± 0.57
Cytoplasmic contents	0.05 ± 0.01
Membrane fraction	4.24 ± 0.23

centrated membrane suspensions were used (initial protein concentration, 5.5–8.0 mg/ml), thereby requiring only a single detergent for optimal protein resolution. The solubilized membrane protein fraction was operationally defined as that which failed to sediment at 45 000 × *g* in 30 min. When the detergent concentration was increased to 2.5%, the membranes were completely solubilized leaving no residue. This preparation was focused in thin layer with 5% acrylamide, 3% bis, 2.3% (v/v) Ampholine ampholytes, and 0.1% (v/v) Triton X-100. The optimal mixture of ampholyte pH ranges producing a linear gradient with uniform conductivity consisted of pH 3.5–10, pH 9–11, pH 5–7, and pH 4–6; increased ampholyte concentration did not enhance resolution. When 140 μg of solubilized membrane protein was focused, 22 bands were visualized in this gradient between pH 3.5 and 9.5 after Coomassie blue staining (Fig. 1). For reference, a schematic diagram of this pattern is presented; the dashed lines indicate bands to faint for photographic reproduction overshadowed by closer more prominent bands.

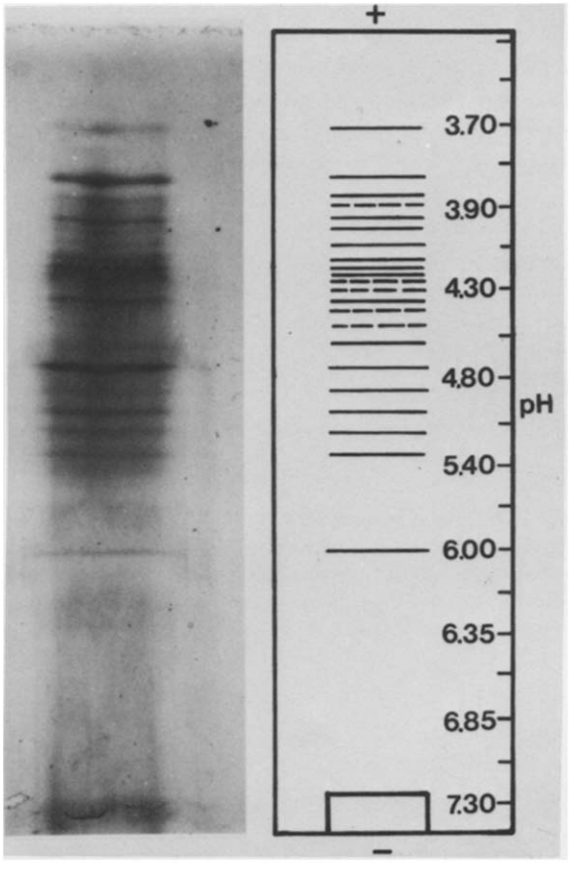

Fig. 1. TLIEF profile of *S. aureus* 6538P membrane proteins. Isolated membranes (5.5 mg protein) were solubilized with 2.5% Triton X-100 and 140 μg protein was focused in a pH range of 3.5–10. The boxed area near the cathode indicates the sample application site (schematic). The dashed lines represent weakly staining membrane protein components. The p*I* values of the components are (from to to bottom): 3.70, 3.85, 3.90, 3.95, 4.00, 4.05, 4.10, 4.15, 4.20, (4.25), (4,30), 4.35, (4.40), (4.45), 4.60, 4.70, 4.80, 4.90, 5.10, 5.30, 6.00.

Under the experimental conditions employed, the majority of components were focused in the acidic region between pH 3.80 and 5.40. One acidic component was found near the anode at 3.70 and a less acidic component was found at 6.00. By inspection, the membrane protein components resolved by this technique appeared to differ in quantity; increasing the amount of protein applied (>140 μg) did not add to the number of components detected. Varying the sample application site between anode and cathode also did not alter the profile. Na basic components were detected between pH 7.0 and 9.5 under these conditions. The electrophoretic profile was unaffected when PMSF (0.1 mM) was included in the buffers used during membrane isolation and solubilization.

Enzymatic activities of membrane proteins after TLIEF

To evaluate the biological activities of the membrane proteins and identify the focused bands as enzymes, the components were analyzed following TLIEF by zymogram methodologies. Under the experimental conditions described, five commonly-classified bacterial membrane-associated proteins displayed enzymatic activity (Table II).

The isoelectric points were determined by taking the pH measurement from the center of the reactive band and represent the results of two separate experiments with four zymograms each. The range of pH units shown indicates the pH measurements obtained from the entire width of the band and includes the slight broadening of the respective enzymatically-reactive area that occurred during development. As TLIEF produces tightly focused bands, the broadening may have occurred during the manipulations following the completion of focusing and processing of

TABLE II

ZYMOGRAM METHODS IN TLIEF

Enzyme (EC number)	p*I* (range)
ATPase (EC 3.5.1.3)	4.20 (4.05–4.40)
NADH dehydrogenase (EC 1.6.99.3)	4.25 (4.20–4.30) 4.35 (4.30–4.40)
Succinate dehydrogenase (EC 1.3.99.1)	4.85 (minor band) 5.10 (4.95–5.15) 5.35 (minor band)
Malate dehydrogenase (EC 1.1.1.37)	3.90 (3.90–3.95)
Lactate dehydrogenase (EC 1.1.1.27)	3.85 (3.80–3.85)
Alkaline phosphatase	Absent

the gel. However, the broadening resulted in a range of pH units of 0.05 at a minimum (lactate dehydrogenase) to 0.35 at a maximum (ATPase). In every zymogram attempted, the omission of the substrate from the reaction mixture failed to result in the respective reactive band when processed, and boiled protein preparations also failed to show reactive bands. Of the enzymes examined, ATPase, lactate dehydrogenase, and alkaline phosphatase had an absolute requirement for added divalent cations (Mg^{2+}, Mg^{2+}, and Zn^{2+}, respectively) in the zymogram reaction mixture.

ATPase activity was located by substrate dependent staining with lead salts at a p*I* of 4.20 (Fig. 2). The intensity of the zymogram reaction varied with the amount of sample applied.

Processing of the focused membrane proteins for NADH dehydrogenase resulted in the identification of two reactive bands (Fig. 3). The bands had isoelectric points of 4.25 and 4.35 suggesting the presence of two discrete NADH dehydrogenase isoenzymes in the staphylococcal membrane from this strain.

The zymogram for succinate dehydrogenase resulted in a major reactive band of p*I* 5.10 (Fig. 4). Two less intense bands of p*I* 4.85 and 5.35 were produced on both sides of the main succinate dehydrogenase band. Varying the cathodic sample application site did not alter the location of these minor succinate dehydrogenase bands.

Enzymatic staining for malate dehydrogenase produced a reactive area with an approximate p*I* of 3.90 (Fig. 5). Using the reaction mixture described, this reactive area appeared light blue against a background which had turned slightly darker upon prolonged incubation at 37°C. Increasing the amount of solubilized membrane protein applied resulted in a corresponding increase in the intensity of the malate dehydrogenase reactive band following zymogram processing.

Lactate dehydrogenase activity was located after zymogram staining with an approximate p*I* of 3.85 (Fig. 6). The LDH reactive area was observed as a sharp line preceded by a diffuse translucent area. The

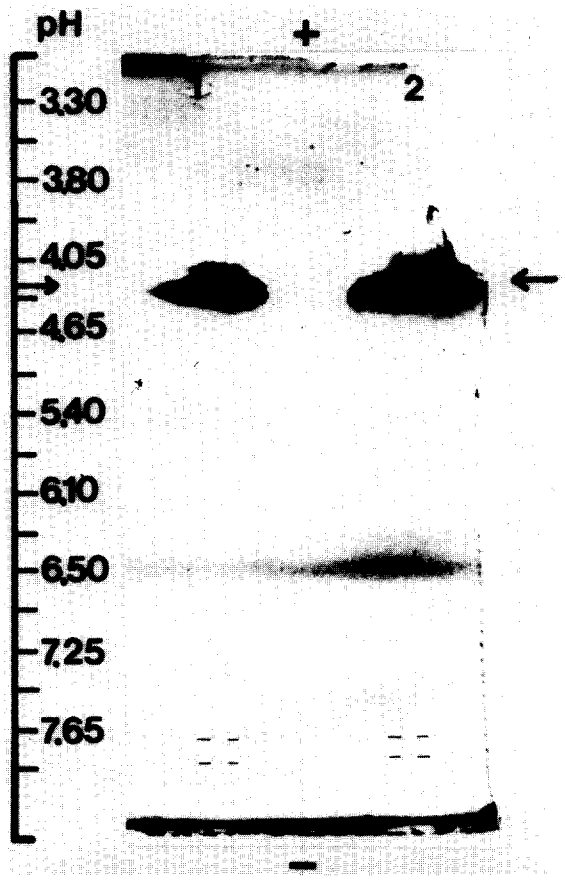

Fig. 2. ATPase zymogram after TLIEF. The ATPase activity can be seen as an enzymatic deposit of PbS (irregular band) with an approximate p*I* of 4.20 (arrow). In every case, the dashed area near the cathode represents the sample application site. Lane 1: 100 μg protein; lane 2: 140 μg protein.

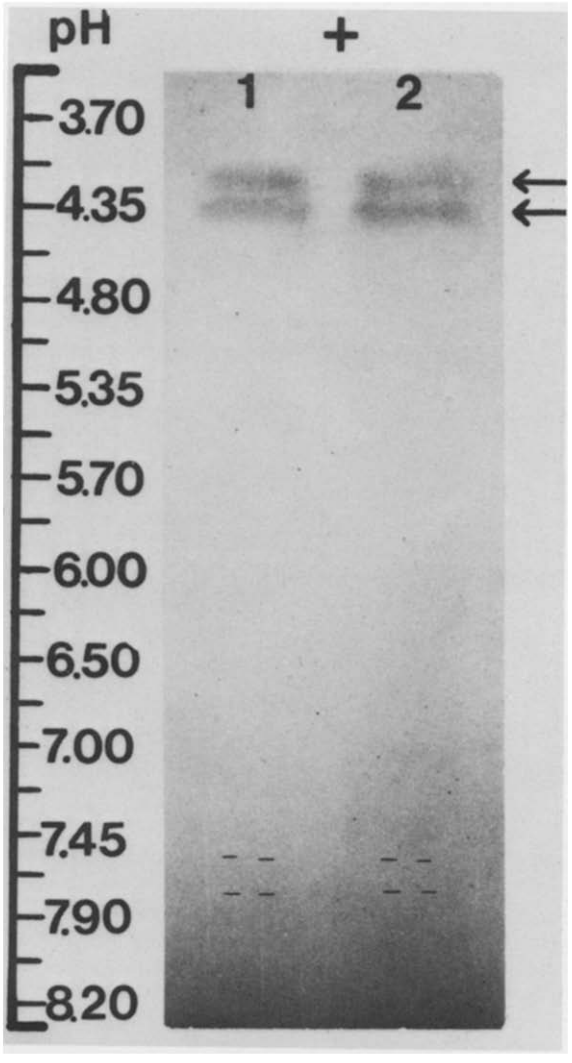

Fig. 3. NADH dehydrogenase zymogram after TLIEF, NADH dehydrogenase activity can be seen as two formazan bands with approximate p*I* values of 4.25 and 4.35. Lanes 1 and 2: 140 μg protein.

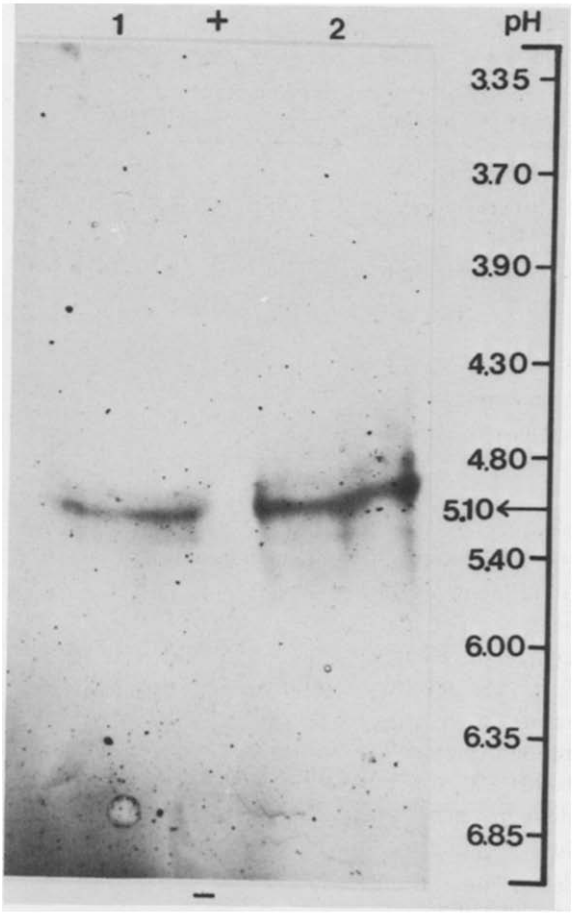

Fig. 4. Succinate dehydrogenase zymogram after TLIEF. The enlarged area of succinate dehydrogenase activity in the gel is seen as a formazan band with an approximate p*I* of 5.10. Lane 1: 100 μg protein; lane 2: 140 μg protein. Two less intense succinate dehydrogenase reactive bands can be seen on each side of the main band in lane 2 with approximate p*I* values of 4.85 and 5.35.

skewed appearance of the band in lane 3 was caused by an uneven cooling resulting in slight band distortion.

Certain authors [14] have reported on the association of the enzyme alkaline phosphatase with other Gram-positive bacterial membranes. The alkaline phosphatase zymogram attempted was based on the action of alkaline phosphatase on the non-fluorescent substrate 4-methylumbelliferone phosphate in an alkaline medium. The liberated 4-methylumbelliferone was then localized by its fluorescence under ultraviolet light at 360 nm. Although a commercial alkaline phosphatase preparation focused under the conditions described demonstrated a fluorescent band with a p*I* of 8.5, the membrane proteins failed to show any reaction.

Each of the enzymatically active bands corresponded with a band and an approximate p*I* value as visualized in the Coomassie blue stained TLIEF profile.

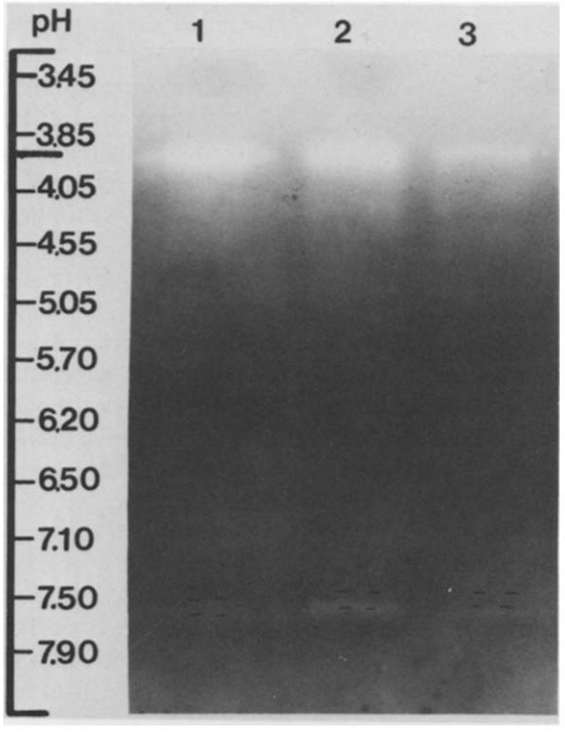

Fig. 5. Malate dehydrogenase zymogram after TLIEF. Malate dehydrogenase activity can be seen as a light banded area with an approximate p*I* of 3.90. Lane 1: 140 μg protein; lane 2: 100 μg protein; lane 3: 75 μg protein. The bands are seen against a dark background which turned darker upon development and after stopping the reaction in acetic acid.

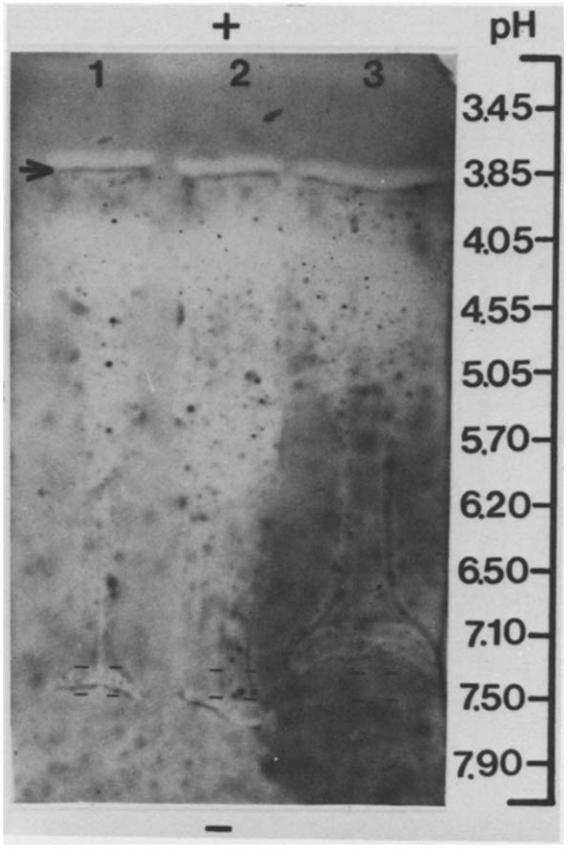

Fig. 6. Lactate dehydrogenase zymogram after TLIEF. Lactate dehydrogenase activity can be seen as a thin formazan band with an approximate p*I* of 3.85. Lanes 1–3: 140 μg protein. Long trails appear from the sample application site (dashed area) leading up to the reactive band.

Discussion

The data presented in this paper demonstrates for the first time the non-denaturing electrophoretic delineation of *S. aureus* membrane proteins. The lysostaphin-induced production of *S. aureus* 6538 P protoplasts permitted the isolation of clean membrane preparations while the analysis of the membrane proteins by TLIEF provided a reproducible method for their resolution, identification as enzymes, and definition of isoelectric points. The enzymatic integrity of the membrane did not seem to be adversely affected by the isolation methods as evidenced by the enrichment of ATPase activity in the membrane fraction without considerable loss during isolation. Similarly, the demonstration of ATPase, NADH dehydrogenase, succinate dehydrogenase, malate dehydrogenase, and lactate dehydrogenase activities by zymogram analyses implied that sufficient conformational integrity was maintained following membrane isolation and solubilization decreasing the possibility of physical disruption or proteolytic destruction. Further, the profile was unaffected when the protease inhibitor was omitted from the purification buffers and solubilization mixture suggesting the absence of PMSF-sensitive proteolytic activity.

The effective solubilization by Triton X-100 confirms the usefulness of this agent as a membrane solubilizer [15,16]. Further data is necessary to determine the nature of detergent binding to specific membrane protein domains as has been suggested previously [16]. The use of dilute *S. aureus* membrane

suspensions to achieve optimal solubilizing potential with Triton X-100 is consistent with observations using Gram-negative bacterial membranes [17]. The clustered appearance of purified *S. aureus* vesicles that we have demonstrated following isolation [2] may contribute to the difficulty of membrane dissolution by the detergent in more concentrated membrane suspensions.

All of the 22 membrane proteins resolved in the pH 3.5–10 gradient were focused with isoelectric points less than 6.0 similar to the largely acidic character of inner membrane proteins from *Escherichia coli* [18]. The conclusion that the membrane proteins were focused to completion, and, consequently, represent an accurate determination of individual isoelectric points, is supported by two observations: (1) application of membrane protein extracts at various locations between electrodes resulted in identical banding patterns; (2) the individual isoelectric points of the focused components did not change with increased focusing times [19]., Also, in this regard, it is generally accepted that the non-ionic character of Triton X-100 does not alter the intrinsic isoelectric points of these proteins [16]. The detergent milieu did not alter the isoelectric points of several water-soluble protein controls treated under the same conditions. The absence of any detectable basic protein components in the TLIEF profile extending to pH 10 would argue against the possibility of ribosomal protein contribution to the membranes as the basic nature of these proteins has been largely established [20]. It has further been shown that RNAase treatment during membrane isolation reduces the amount of RNA (orcinol-reactive material) from isolated membrane preparations [2,21].

The presence of multiple bands exhibiting enzymatic activity was observed for two membrane enzymes: NADH dehydrogenase and succinate dehydrogenase. Owen and Salton [6] have reported *Micrococcus lysodeikticus* NADH dehydrogenase activity partitioning between membrane and cytoplasm, in addition to a distinct activity lozalized exclusively in the membrane. While we have not examined the possibility of a cytoplasmic NADH dehydrogenase associating with the membrane fraction, the extensive washes performed on the isolated membrane preparations reduces such non-specific association with the membrane.

The failure to detect detergent solubilized membrane alkaline phosphatase activity by zymogram analysis lends support to periplasmic or cytoplasmic origin of this enzyme [22]. The proteins observed by TLIEF most likely represent intrinsic membrane proteins [23] since they were not released following a wash in high salt buffer and detergent solubization was necessary for their resolution.

We have described the heterogeneity of homogeneous *S. aureus* 6538P membrane proteins by isoelectric focusing. The description of individual isoelectric points coupled with the partial enzymatic identification of certain membrane proteins should assist in describing the role of these proteins in overall membrane function and in the binding of different agents to the staphylococcal membrane.

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