Biochimica et Biophysica Acta, 637 (1980) 189–201
Elsevier/North-Holland Biomedical Press

BBA 48105

ELECTROPHORETIC PROFILES OF CYANOBACTERIAL MEMBRANE POLYPEPTIDES SHOWING HEME-DEPENDENT PEROXIDASE ACTIVITY *

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(Received 5th January, 1981)

Key words; Polypeptide profile; Photosynthetic membrane; Heme dependence; Peroxidase; (Cyanobacteria)

The photosynthetic membranes of *Anacystis nidulans* R2 were examined electrophoretically following solubilization with lithium dodecyl sulfate. Electrophoresis yielded six prominent chlorophyll-containing bands. In addition, five polypeptides were observed which possessed heme-dependent peroxidase activity, monitored by incubating gels with 3,3′,5,5′-tetramethylbenzidine plus hydrogen peroxide. One such polypeptide, at 105 kdaltons, was removed by repeated washing of the membranes. Four remaining peroxidase-active polypeptides were observed at 7.2, 13.5, 18.5 and 33 kdaltons. Further examination of these four polypeptides yielded the following results. (1) The mobility of the 33 kdalton polypeptide was altered from 29 to 33 kdaltons upon heating (70°C) during membrane solubilization. (2) All four polypeptides showed stable heme-protein associations in the presence of 8 M urea; however, in the presence of urea, alterations in protein mobility were observed for each polypeptide and only two (at 13.5 and 33 kdaltons) showed peroxidase activity following heating (70°C) during membrane solubilization. (3) The presence of thiols during membrane solubilization at 0°C was required to observe peroxidase activity at 7.2 kdaltons. These results, when compared to known properties of isolated cytochromes, suggest that the four polypeptides characterized here correspond to the subunits of photosynthetic cytochromes. Electrophoretic assessment of maize mutants lacking cytochrome *f* and b_6 activity supports this suggestion.

Introduction

Thylakoid membranes obtained from unicellular cyanobacteria have been shown to contain in excess of 50 membrane polypeptides when analyzed by dodecyl sulfate polyacrylamide gel electrophoresis [1]. The identities of these polypeptides, however, remain largely unknown and, thus far, characterization has proceeded along three paths. First, several polypeptides have been shown to be altered in mutants which display defective photosynthetic activity [2,3]. For example, an alteration of a peptide from 53 to 51 kdaltons was found in a mutant possessing aberrant Photosystem II activity [1,2]. Second, electrophoretic assessment of Photosystem I- or Photosystem II-enriched membrane fragments, prepared by digitonin treatment, has established the molecular weight classes of peptides associated with each photosystem [4]. Third, the use of probes which label either surface-exposed or lipophilic regions of membrane proteins has elucidated the topological position within the cyanobacterial thylakoid of many polypeptides [1].

Identification of specific bands on an electrophoretic profile has relied heavily upon a correlation with known molecular weights of isolated, characterized membrane proteins. For example, Sadewasser

* This work was presented in preliminary form at the Midwest Photosynthesis Meetings, October 26–27, 1980.

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Abbreviations: LDS, lithium dodecyl sulfate; TMBZ, 3,3′, 5,5′-tetramethylbenzidine; Tricine, *N*-tris(hydroxymethyl)-methylglycine.

0005-2728/81/0000–0000/$02.50

and Sherman [1] suggested that five EDTA-extractable, surface-labeled polypeptides corresponded to the subunits of the coupling factor enzyme of cyanobacteria [5,6]. Such correlations are complicated by the fact that the binding of dodecyl sulfate to many hydrophobic proteins is unpredictable [7]. Thus, for certain membrane polypeptides, electrophoretic mobility is not a reflection of molecular weight and alternative approaches must be found to establish their identities. Indeed, the ability to analyze the functional attributes of certain proteins directly on acrylamide gels would be a major aid to the ultimate understanding of membrane structure and topography.

This report presents an electrophoretic examination of the thylakoid membranes of the cyanobacterium, *Anacystis nidulans* strain R2. The focus of this report is a localization of heme-dependent peroxidase activity on electrophoretic patterns of solubilized membranes. Peroxidase activity, localized by the method of Thomas et al. [8] using TMBZ + H_2O_2, is thought to be due to the heme cofactors of proteins such as cytochromes. Five polypeptide bands were observed to possess peroxidase activity following acrylamide gel electrophoresis of *A. nidulans* membranes. Evidence will be presented which suggests that four of these peptides, corresponding to molecular masses of 33, 18.5, 13.5 and 7.2 kdaltons, might have a direct relationship with the polypeptide subunits of cytochromes in photosynthetic membranes.

Materials and Methods

Cells of *A. nidulans* strain R2 were grown axenically in liquid culture as previously described [3] and were routinely monitored for bacterial contamination both microscopically and by plating on nutrient agar. This strain of *A. nidulans* was originally isolated in Russia and was kindly provided by Dr. G. Van Arkel, University of Utrecht. Cells were harvested by centrifugation and were washed and concentrated in buffer A, consisting of Tricine (20 mM, pH 7.5), mannitol (0.5 M), and $NaKHPO_4$ (5 mM). Following the addition of 10 mM EDTA, spheroplasts were obtained by the incubation of cells (37°C) with 10 mg/ml lysozyme (Sigma). Spheroplast preparation was monitored microscopically and the incubation was terminated when a rounding of over 95% of the rod-shaped cells was obtained (approx. 2 h). Spheroplasts were centrifuged, washed in buffer A, and osmotically ruptured by resuspension in 10 mM Tricine. The thylakoid membrane fraction was collected by centrifugation and was both washed and resuspended in buffer A to a final chlorophyll concentration [9] of about 100 μg/ml. Maize thylakoids were prepared as previously described [10].

Photosystem II and Photosystem I membrane fractions were prepared by fractionating membranes with digitonin followed by sucrose gradient centrifugation [4]. Purification was obtained by passage of the fraction through a DE-23 column as previously described [4]. We also found that relatively purified Photosystem II-enriched membrane fractions could be prepared by incubation of thylakoid membranes with a low concentration of *N*-tetradecyl-*N*,*N*,-dimethyl-3-ammonio-1-propanesulfonate (Zwittergent 3-14, Calbiochem Behring Corp.). Membranes were suspended in buffer B (10 mM Tricine, pH 7.5; 10 mM KCl, 10 mM EDTA) to a concentration of 10 mg protein/ml [11]. The zwitterionic detergent was added to a final concentration of 0.5 mg/ml, and, following 2 h stirring on ice, the mixture was centrifuged (1000 × *g* for 5 min) to remove unresolved membranes. Sucrose density gradient centrifugation (5–20% sucrose in buffer B plus 0.5 mg/ml detergent) for 16 h at 20 000 rev./min (Beckman L265B ultracentrifuge equipped with an SW-27 rotor) yielded two distinct bands. The top band possessed about 7% of the total chlorophyll and contained predominantly Photosystem II activity as monitored photochemically and by low-temperature spectrofluorimetry [4].

Electrophoretic assessment of the protein composition of thylakoid membranes was accomplished by the methods of Delepelaire and Chua [12] using the buffer system of Laemmli [13]. By this method, gels were cast having varied polyacrylamide gradients with a 5% polyacrylamide stacking gel. Gels were run at constant power (2.75 W) in the cold (approx. 4°C). All gels were prerun for 30–45 min prior to loading of the samples. Samples were prepared for electrophoretic assessment by resuspending membranes to 8 mg protein/ml in 10 mM Tricine plus 6% sucrose. Dithiothreitol, when present, was 30 mM. To this suspension, LDS (Gallard-Schlesinger Chem. Corp.) was added to a final concentration of 1% and samples

were either held on ice or were heated in a water bath to the appropriate temperature for 10 min prior to loading on a gel. All gels were loaded with 50–80 μg protein per well and the marker proteins which were coelectrophoresed with each gel were: myosin (200 000), phosphorylase *a* (97 000), bovine serum albumin (67 000), ovalbumin (45 000), chymotrypsinogen (25 000), cytochrome *c* (12 000) and insulin (3000 daltons).

Observations suggested that the gel system described above adequately resolved only those peptides in a size range above 12 kdaltons (see Fig. 2A). Presumably, this is due to a complex set of interactions involving both the binding of dodecyl sulfate to small, hydrophobic polypeptides [7,14] and interference in the lower molecular weight range by lipid/detergent mixed micelles. Unfortunately, one intriguing polypeptide was observed between 5 and 8 kdaltons which possessed heme and which required further investigation. Thus, to investigate the polypeptide profile between 5 and 8 kdaltons, we employed a gel system modified from that of Swank and Munkres [14]. As shown in Fig. 2B, this modified system allowed the resolution of polypeptides in this range. Polyacrylamide gradient gels containing 8 M urea were cast as above, except that the buffer system used throughout was 0.1 M H_3PO_4 neutralized to pH 7.2 with Tris base (Sigma). Sodium dodecyl sulfate (0.1%, from BioRad) was included in the running buffer. Samples were prepared as above except that 8 M urea was included in the solubilization mixture.

Upon termination of the electrophoretic run (approx. 7–9 h), gels were photographed through a blue filter to record chlorophyll-associated banding patterns. Peroxidase activity [10] was localized by incubation of the gel with 0.5 mg/ml TMBZ (Sigma) in 50% methanol, 1 M sodium acetate (pH 4.7) for 30 min, followed by the addition of H_2O_2 to 0.5%. It was essential that the incubation be carried out in the dark owing both to the lability of TMBZ and to the photochemical oxidation of TMBZ by chlorophyll. Staining occurred within 45 min and stain could be removed with Na_2SO_3 [8]. Coomassie brilliant blue R-250 (BioRad) staining was accomplished as previously described [12].

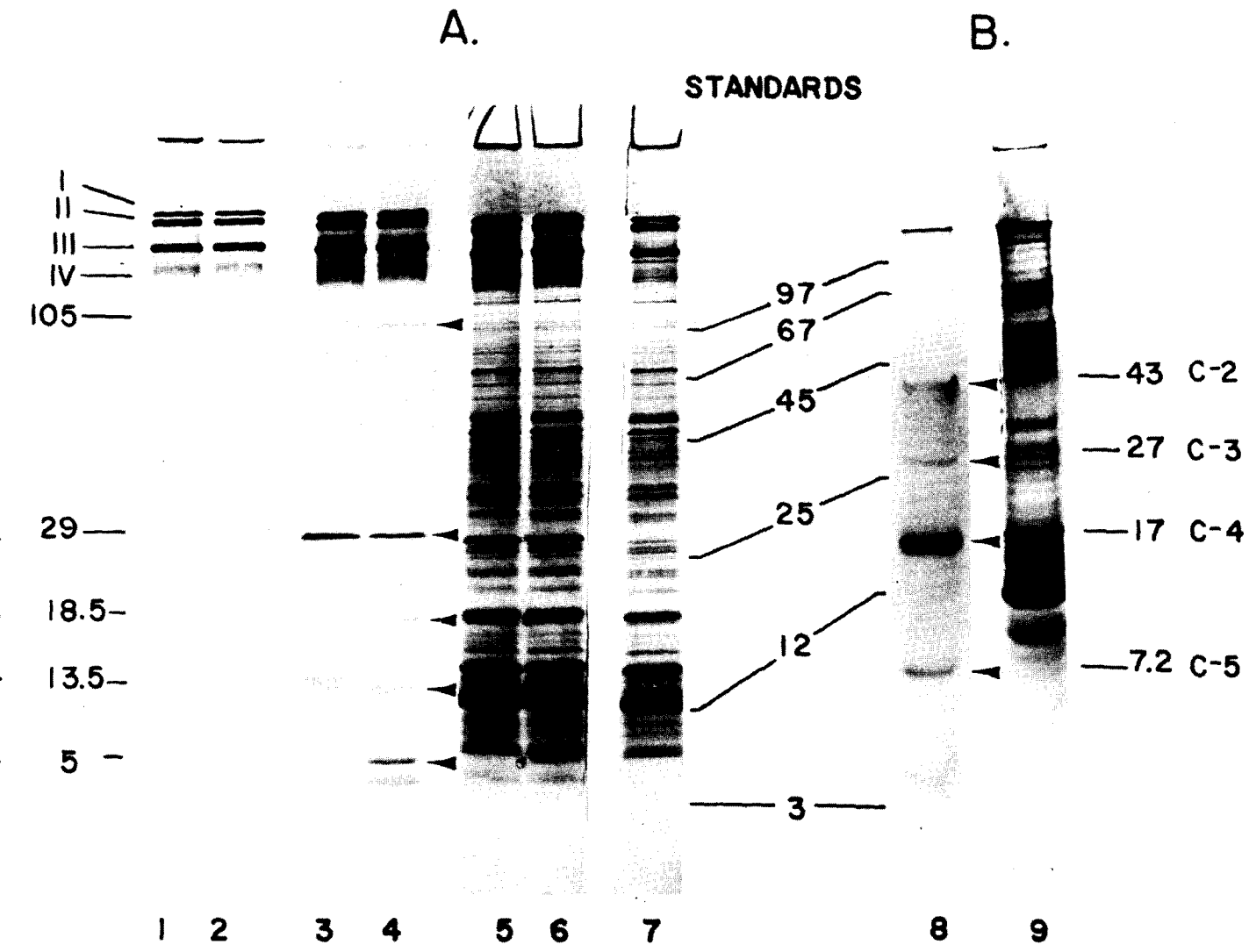
Results and Discussion

Previous studies investigating cyanobacterial membrane structure have combined an electrophoretic assessment of membrane composition with detergent fractionation [4], site-specific polypeptide labeling [1], and a study of photosynthetically deficient mutants [2,3]. We have extended these studies on cyanobacterial thylakoid composition with the use of an LDS-polyacrylamide electrophoretic system [12]. Use of this detergent permits electrophoresis at reduced temperatures (4°C), allowing a retention of certain protein-lipid or protein-ligand hydrophobic interactions. This confers two major advantages on our studies. First, as shown in Fig. 1, this system permits the study of chlorophyll-protein association in photosynthetic membranes. Second, and more germane to the focus of this report, this system allows us to visualize directly on gels the heme-containing subunits of photosynthetic cytochromes. This feature will be discussed in detail below. Fig. 1 shows that, depending upon the mode of membrane sample preparation, six prevalent chlorophyll-protein aggregates were routinely observed with these membranes. In contrast, with studies of higher plant thylakoids (cf. Fig. 5) [12,15], and in agreement with other work using cyanobacteria [16,17], a majority of the chlorophyll was found in aggregates of high molecular weight (Fig. 1, bands I–III). Heating (30°C) of membrane samples during solubilization with LDS (lane 2) caused the appearance of a fourth chlorophyll-associated band (band IV) at the expense of aggregates of higher molecular weights. The corresponding changes in the protein patterns upon heating parallel the changes observed with the chlorophyll patterns and will be discussed in greater detail below.

Interestingly, two chlorophyll-associated protein bands were evident in this electrophoretic system (Fig. 1, bands V and VI) which were exceedingly labile. Chlorophyll was removed from these bands either by heating during membrane solubilization or migration of these bands through a greater acrylamide concentration than that employed in Fig. 1. Band VI often appears as a doublet (47 kdalton band of Fig. 1, lane 3). In addition, the top band of this doublet was found to be enhanced in preparations enriched in Photosystem II activity. This enhancement was observed with Photosystem II-enriched membrane frac-

Fig. 1. Six chlorophyll-protein aggregates observed with membranes and submembrane fractions of cyanobacteria. Samples were solubilized at 0°C (lanes 1, 3–5) or at 30°C (lane 2) and were electrophoresed on a 5–15% polyacrylamide gradient. The lanes contained: 1 and 2, membranes; 3, Photosystem II particles; 4 and 5, Photosystem I particles. Samples containing 80 μg protein were loaded in all lanes except lane 5; lane 5 is an inset of a lane similar to lane 4 except that lane 5 was purposely overloaded (with 160 μg protein) to enhance the appearance of minor chlorophyll bands. I–VI denote chlorophyll-containing bands whereas FP denotes free pigment. Positions of marker proteins are given in kdaltons.

tions prepared both by the method of Newman and Sherman [4] (Fig. 1) and by the use of Zwittergent 3-14 (data not shown). Such preparations also exhibited a predominance of band III. In contrast, band V was never observed in appreciable amounts associated with Photosystem II fractions and was found in association with Photosystem I (Fig. 1, lanes 4 and 5). Thus, there appears to be a clear delineation between



Chlorophyll-protein, TMBZ-stained and polypeptide profiles of *Anacystis* membranes on gels containing LDS alone (A) or with 8 M urea (B). Samples
ized at 0°C (10 min) and were electrophoresed on 10–20% polyacrylamide gradient gels. (A) The lanes shown: 1 and 2, chlorophyll-banding pattern sh
phyll-protein bands I–IV; 3 and 4, blue bands C-1–C-5 (marked with arrow heads) which appear after TMBZ + H_2O_2 staining; 5 and 6, Coomassie blue
er staining with TMBZ + H_2O_2; 7, Coomassie blue profile alone. Note that in lanes 1, 3 and 5, samples were solubilized without thiols. In contrast, lanes
7 contain material solubilized in the presence of 30 mM dithiothreitol. (B) The lanes show: 8, blue bands C-2–C-5 (arrow heads) which appear after TM
staining; 9, Coomassie blue profile alone. Samples in B were solubilized in the presence of 30 mM dithiothreitol. The molecular masses of bands C-1–C-
n kdaltons relative to marker protein standards.

bands V and VI with respect to their association with photosystem particles following detergent fractionation. This difference has both structural and functional implications for the association of these bands within the photosynthetic apparatus.

A second major advantage bestowed by the LDS-polyacrylamide electrophoretic system is the localization of hydrophobic protein-ligand interactions, such as the identification of heme-dependent peroxidase activity by the protocol of Thomas et al. [8]. As shown in Fig. 2A, peroxidase activity was localized by the incubation of gels with TMBZ plus peroxide, causing a heme-catalyzed oxidation of TMBZ and a precipitation within the gel of the TMBZ semiquinone. This precipitate, a brilliant blue derivative of Benzidine-blue [18,19], was clearly distinguishable from the green chlorophyll bands. In addition, illumination of gels following removal of excess peroxide allowed resolution of minor chlorophyll-containing bands owing to a chlorophyll-dependent photooxidation of the dye.

Fig. 2 presents a series of electrophoretic profiles which demonstrate the TMBZ-oxidation patterns found using cyanobacterial membranes. Membrane samples were solubilized with LDS (at ice temperatures) both in the presence (lanes 2, 4 and 6–9) and absence (lanes 1, 3 and 5) of thiols. Assessment of membrane samples using LDS-polyacrylamide gels (Fig. 2A) and a sequential staining protocol showed the following. First, lanes 1 and 2 show the chlorophyll-protein patterns which were evident before staining. Second, five bands appear following staining with TMBZ + H_2O_2 (lane 4). These bands, designated C-1–C-5 in Fig. 2A, range in apparent molecular mass from 5 to 105 kdaltons. In addition, these bands differ in sensitivity to the presence of thiols during membrane solubilization (cf. lanes 3 and 4) and the peroxidase activity of these bands was severely inhibited by preincubation of gels with azide prior to TMBZ staining (data not shown).

Two TMBZ-stained bands, C-1 and C-5, are particularly notable since they differed from the remaining three bands in several respects. For example, C-1 (at 105 kdaltons) apparently represents a peripheral membrane protein which is easily removed from the membranes. Repeated washings of thylakoids removed this polypeptide from the membrane fraction. In addition, the osmotic rupture of spheroplasts during thylakoid isolation released a TMBZ-staining polypeptide of similar molecular weight into the soluble fraction (data not shown).

Band C-5, on the other hand, was the most labile of the bands which possessed peroxidase activity. As shown in Fig. 2A (lanes 3 and 4), the presence of dithiothreitol was required during the treatment of membranes with LDS to resolve optimally band C-5. This is in marked contrast with the behavior of the other four bands, which were relatively insensitive to the presence of thiols during membrane solubilization.

A comparison of TMBZ-staining with Coomassie blue incorporation at the 5 kdalton region (Fig. 2A, lanes 4–6) reveals the sensitivity of the TMBZ-staining protocol. Although band C-5 clearly possessed peroxidase activity (lane 4), staining with Coomassie blue alone showed no detectable band at a corresponding position (lane 6). Poor Coomassie blue incorporation most probably reflects both the low amount of protein in this region and the poor resolution of the LDS gel system below 12 kdaltons [14]. Thus, to show a correspondence of protein with TMBZ oxidation and to obtain a more reliable estimate of the size of this low molecular weight band, we reexamined the thylakoid polypeptide profile using an electrophoretic gel system containing 8 M urea modified from that of Swank and Munkres [14]. In the presence of this chaotropic agent, peroxidase activity was located at 7.2 kdaltons (Fig. 2B). In addition, Coomassie blue incorporation was clearly observed at this region.

Three bands were stained with TMBZ in the region between 13 and 35 kdaltons (Fig. 2A). These polypeptides (C-2–C-4), which migrated in the molecular mass regions of 29, 18.5 and 13.5 kdaltons, are intrinsic components of the thylakoid membrane. Repeated washings of thylakoids had little effect on the staining activity observed for these bands. When membrane solubilization was performed at 0–4°C, the staining patterns observed were insensitive to the concentrations of either thiols or LDS. In addition, during the preparation of Photosystem II-enriched membrane fragments using either digitonin [4] or Zwittergent 3-14, bands C-2 and C-3 were observed as contaminants prior to purification of the Photosystem II fraction by column chromatography (data not shown).

Interestingly, bands C-2–C-4 exhibited a some-

what different staining pattern when examined using the urea-containing gel system of Fig. 2B. These differences demonstrate both the difficulty in interpreting comparative electrophoretic results and the value of a staining technique to visualize the functional attributes of specific membrane peptides directly on gels. As shown in Fig. 2B, four polypeptides were observed which possessed peroxidase activity, band C-1 having been removed by washing the membranes during sample preparation. The four bands correspond to bands C-2–C-5 of Fig. 2A, yet bands C2–C-4 showed marked decreases in mobility relative to marker proteins. Mobility changes of 29 to 43, 18.5 to 27, and 13.5 to 17 kdaltons were apparent for C-2–C-4, respectively, upon a comparison of the LDS and urea gel systems. It is doubtful that the latter size measurements, obtained using the urea system, are more accurate estimates of the molecular weights of these bands. The LDS system of Fig. 2A has yielded polypeptide profiles (above 12 kdaltons) which favorably correspond with polyacrylamide gel electrophoresis under totally denaturing conditions. In addition, further denaturation induced by heating (70°C, 10 min) in the presence of urea (as in Fig. 2B) yielded changes in the mobilities and the staining profiles of C-2–C-5 (data not shown). Only two bands, C-2 and C-4, showed TMBZ/staining which was stable to such heating and both showed an increase in mobility following this further denaturation. The heat-induced mobility changes were 43 to 39 kdaltons for C-2 and 17 to 14.5 kdaltons for C-4 (data not shown). Thus, there apparently is a difference in the stability of peroxidase activity among bands C-2–C-5. This difference depends upon the stability of a heme-protein association and might aid in discriminating between the mode of heme attachment to each polypeptide (see below).

Heating of membrane samples during solubilization with LDS yielded several specific changes in an electrophoretic profile run under conditions similar to those in Fig. 2A. Prominent among these changes was the breakdown of higher molecular weight chlorophyll-protein aggregates; this was already shown in Fig. 1, where sample solubilization at 30°C caused an appearance of an additional chlorophyll-protein band (IV) at the expense of bands I–III. Fig. 3 shows the effects of heating (70°C, 10 min) both on the TMBZ-staining activity (A) and on the polypeptide profiles (B) of cyanobacterial thylakoids. Heating caused the breakdown of most of the chlorophyll-protein aggregates, with chlorophyll migrating as the free pigment. Interesting changes in the polypeptides profile paralleled the destruction of the chlorophyll-protein aggregates; notably, major increases in polypeptide banding were observed at 71, 46 and 14 kdaltons following heat treatment.

The impact of heating during membrane solubilization on TMBZ-staining activity depended upon the presence of thiols. As shown in Fig. 3, though TMBZ oxidation was stable to heating in the absence of dithiothreitol, inclusion of thiols during treatment with LDS at 70°C destroyed this activity. Fig. 3 also shows that TMBZ staining at 29 kdaltons disappeared upon heating, with a new band appearing at 33 kdaltons; a similar alteration appeared in the Coomassie blue-stained patterns. Two alternative interpretations could explain the changes observed between 29 and 33 kdaltons in response to heating. First, heating could cause a dissociation of heme from the polypeptide at 29 kdaltons. Such a dissociation could result both in a faster migration of the apoprotein and a non-random association of the released heme with a second peptide at 33 kdaltons. Alternatively, the 29 kdalton polypeptide could suffer a configurational or a compositional alteration upon heating, an event which could lessen its mobility in an electrophoretic system. Both interpretations would explain the results of Fig. 3.

These alternatives were evaluated by a reelectrophoresis of the protein which banded at C-2. Fig. 4 shows comparative profiles of membranes (A) and band C-2 (B) which had been excised from a preparative gel. An identical 29 to 33 kdalton shift was observed upon heating (70°C, 10 min) of either membranes or excised band C-2. This shift could be monitored both by Coomassie blue staining and by TMBZ staining. These results demonstrate that the 29 to 33 kdalton shift at C-2 is not due to migration of heme from the protein. Rather, the polypeptide itself suffers a configurational change, due perhaps to increased dodecyl sulfate binding, which yields an altered mobility of 33 kdaltons. This value probably represents a more accurate estimate of the molecular weight of this peptide.

Several arguments, we believe, effectively suggest that the TMBZ-staining patterns observed here repres-

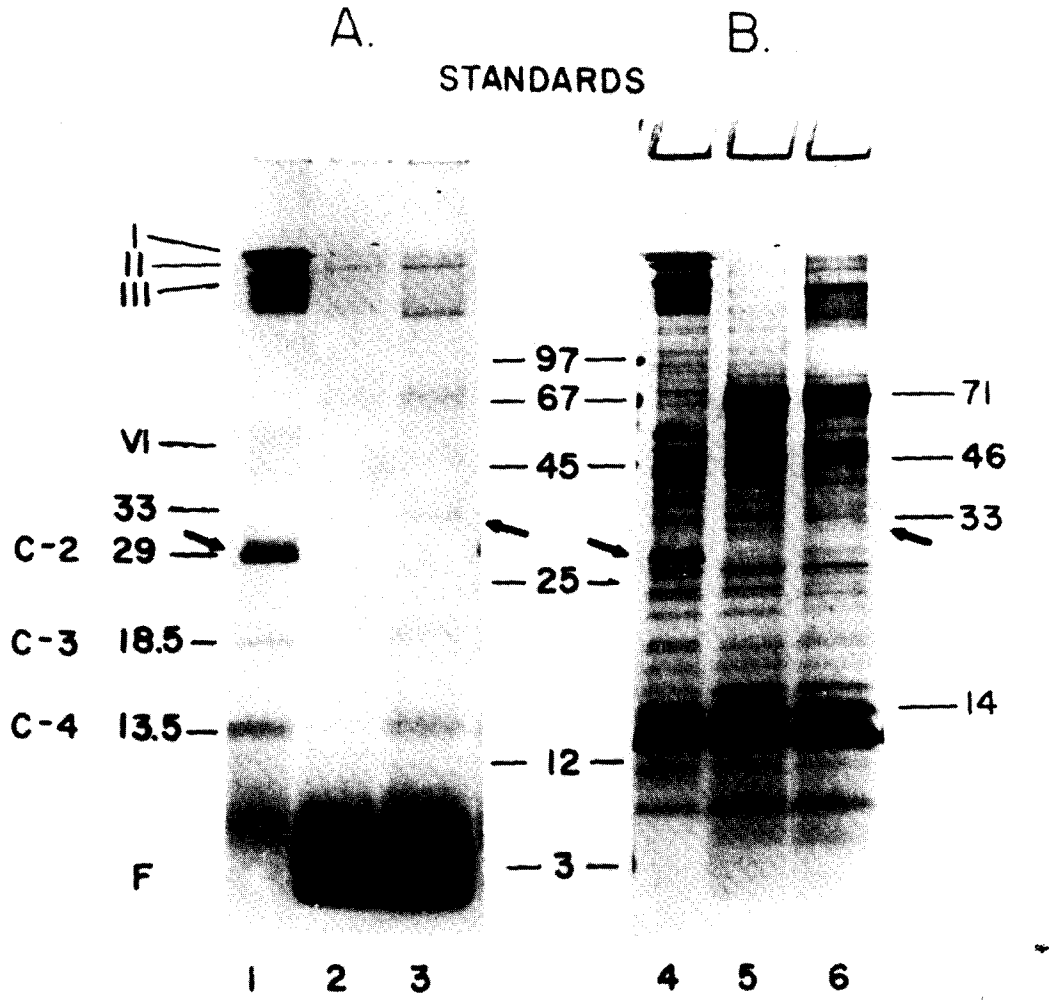


Fig. 3. Effects of solubilization temperature and thiols on the TMBZ + H_2O_2-stained (A) and polypeptide (B) profiles of *Anacystis* membranes. Samples were solubilized for 10 min with 1% LDS as follows: 1 and 4, at 0°C plus dithiothreitol (30 mM); 2 and 5, at 70°C plus dithiothreitol; 3 and 6, at 70°C without thiols. (A) Stained with TMBZ + H_2O_2. The profile shows both chlorophyll-containing bands (I–VI) and the blue bands stained with TMBZ (C-2–C-4). Free pigment (F) apparently interferes with staining at C-5. Note that C-2 shifts in molecular mass to 33 kdaltons upon heating, as denoted by arrows. (B) Same as A, followed by staining with Coomassie blue. The molecular masses of protein increases observed upon heating are noted. Samples were assessed on a 10–18% polyacrylamide gradient gel. The positions of marker protein standards are given in kdaltons.

ent a physiologically significant association of heme with protein. First, oxidation of TMBZ in the presence of H_2O_2 is presumed to depend upon the association of heme with protein. Such an association has been demonstrated in other systems, such as with cytochrome *P*-450 [8] and with cytochrome oxidase [20]. In addition, while this report was in preparation, Hoyer-Hansen [21] showed TMBZ staining associated with electrophoretic profiles of barley thylakoids. This peroxidase activity was restricted to peptide bands containing ^{14}C-labeled products of precursors in prophyrin biosynthesis. Thus, in every system assessed with TMBZ + H_2O_2, staining has arisen due to a heme-protein association.

Second, reproducible TMBZ-staining patterns were observed under a variety of denaturing and partially denaturing conditions. As shown in Table I, these conditions included: (1) treatment with 1% LDS plus

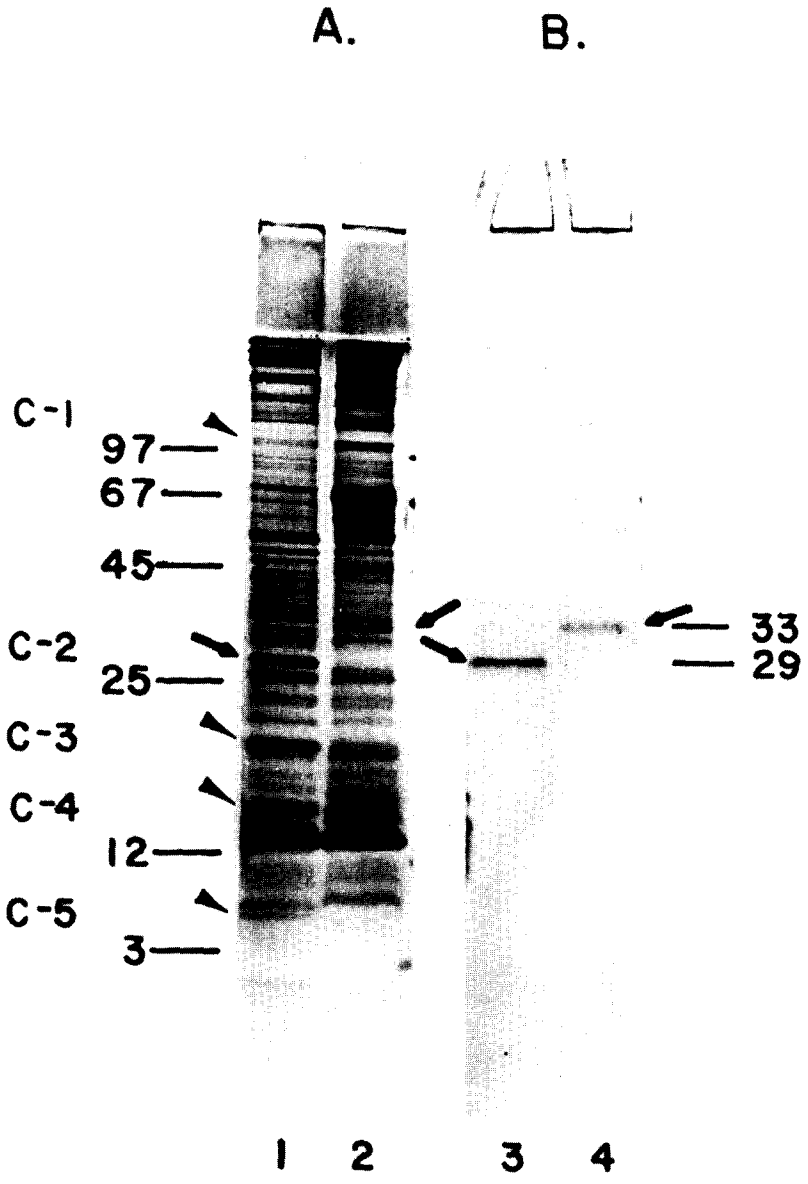


Fig. 4. Effects of solubilization temperature on the profiles of *Anacystis* membranes (A) and band C-2 (B) excised from a preparative gel. Gels were run as in Fig. 2A. (A) Membranes were solubilized (10 min) at either 0°C plus dithiothreitol (30 mM) (lane 1) or 70°C without thiols (lane 2). The gel was first stained with TMBZ + H_2O_2, followed by Coomassie blue. Arrow heads show bands C-1–C-5 and arrows indicate the shift in mobility of C-2 after heating. (B) Band C-2 at 29 kdaltons was excised from a preparative gel, homogenized, and extracted from acrylamide into 10 mM Tricine, pH 7.5, 6% sucrose and 4% dodecyl sulfate. The extract was kept at 0°C (lane 3) or was heated (70°C, 10 min) prior to electrophoresis (lane 4). The gel was stained with Coomassie blue. Arrows depict the 20 to 33 kdalton shift at C-2 upon heating. The positions of marker proteins are given in kdaltons, on this 10–18% acrylamide gel.

thiols; (2) heating in the presence of LDS without thiols, and (3) treatment with 1% LDS plus 8 M urea. In addition, only one band, C-5, was sensitive to thiols in the presence of LDS (see Fig. 2B) and two bands, C-2 and C-4, were stable to heat in the presence of LDS + urea. Thus, the association of heme with protein must be somewhat stable despite the expectation [8,21] that a noncovalent attachment of heme to such complex proteins as the *b*-type cytochromes would be labile. Perhaps a difference between *b*- and *c*-type cytochromes could explain the observation that only C-2 and C-4 are stable to heating in the presence of 8 M urea.

Finally, the molecular weights of isolated photosynthetic cytochromes correspond favorably to the apparent size of bands C-2–C-5. Thus, the photosynthetic cytochromes of cyanobacteria represent an obvious starting point in assessing the identities of the TMBZ-staining polypeptide bands. Two *c*-type cytochromes, cytochrome *f* [22] and cytochrome *c*-552 [23,24], have been isolated from cyanobacteria. Böhme et al. [22] have characterized cytochrome *f* from *Spirulina platensis* and have shown that this protein, with an extremely acidic pH, consists of a single polypeptide chain of 33–34 kdaltons. Conversely, another *c*-type cytochrome (*c*-552) has been isolated from several cyanobacteria [23,24]. The size of this polypeptide, 11–15 kdaltons, is considerably less than that observed for cytochrome *f*. Two *b*-type cytochromes, b_6 [25] and *b*-559 [4,25,26], can be spectrally resolved in cyanobacterial membranes. Both *b*-type cytochromes, when isolated from eukaryotic systems, are complex proteins, as recently summarized by Wasserman [27]. Cytochrome b_6, for instance, is a multimeric protein having four subunits per heme with apparent molecular masses of 20, 15 and 6.6 kdaltons. Cytochrome *b*-559, on the other hand, consists of eight nonidentical subunits of 5.7 kdaltons. In addition, Wasserman [27] suggests that cytochrome *b*-559, in marked contrast with cytochrome b_6, is stabilized by thiols – an interesting suggestion in light of the results of Fig. 2 which show that the appearance of band C-5 was dependent upon the presence of dithiothreitol during membrane solubilization.

An interpretation of our results which is consistent with the molecular weight information cited above is shown in Table I. This interpretation suggests that bands C-2 and C-3 correspond to cytochrome *f* and the large subunit of cytochrome b_6, respectively. In addition, it is possible that band C-4 at 13.5 kdaltons represents a *c*-type cytochrome which is thought to replace plastocyanin under certain cell growth conditions [23,24]. This interpretation could also explain the differences in TMBZ-staining stability when membrane samples are heated in the presence of 8 M urea; only bands C-2 and C-4 showed this enhanced stability and most probably correspond to the two photosynthetic *c*-type cytochromes.

At present, we are unable to distinguish between

TABLE I

COMPARISON OF THE OBSERVED MOLECULAR WEIGHTS OF BANDS C-2–C-5 WITH MOLECULAR WEIGHTS OF ISOLATED PHOTOSYNTHETIC CYTOCHROMES

Observed molecular masses are summarized from data of Fig. 2 or as given in text. NH, samples solubilized at 0°C; H, samples heated (70°C) for 10 min during solubilization. Molecular mass of isolated cytochrome is given in kilodaltons. References given in text.

Band	Observed molecular mass (kdaltons)				Stable to heat + 8 M urea	Requires thiol at 0°C	Presumed origin	Molecular mass of isolated cytochrome
	Dodecyl sulfate system		Urea system					
	NH	H	NH	H				
C-2	29	33	42	39	+	–	cytochrome *f*	33–34
C-3	18.5	18.5	27	–	–	–	cytochrome b_6	20
C-4	13.5	13.5	17	14.5	+	–	cytochrome *c*-552	11–12
C-5	5	5	7.2	–	–	+	cytochrome *b*-559 or b_6	5.7 or 6.6

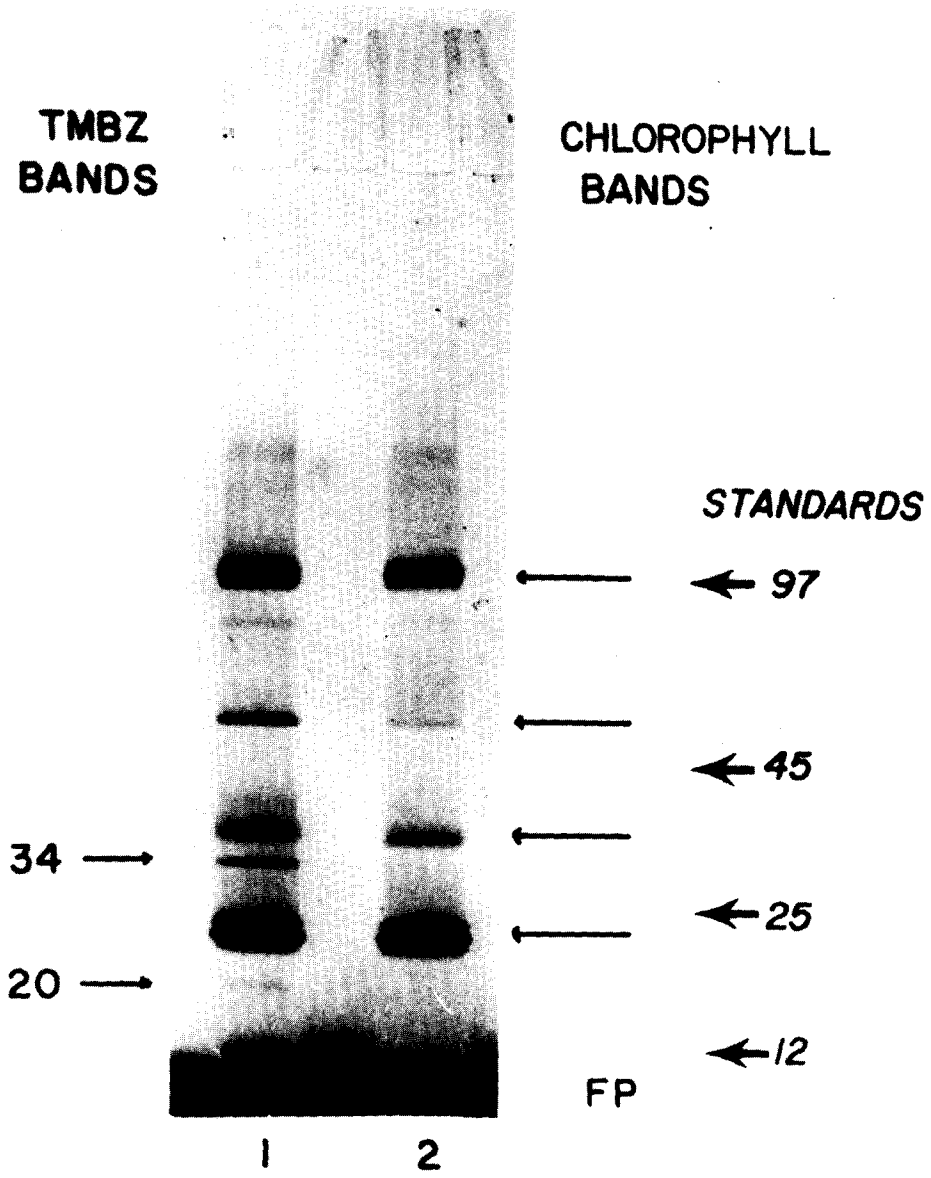


Fig. 5. Chlorophyll-protein bands and TMBZ + H_2O_2-stained profiles of thylakoids from maize wild-type (lane 1) and mutant *hcf*-3 (lane 2). Membranes were solubilized at 0°C (10 min with 1% LDS and were electrophoresed on a 5–15% polyacrylamide gradient gel similar to that of Fig. 1. The gel was stained with TMBZ + H_2O_2. Chlorophyll-containing bands are shown by arrows on the right. TMBZ bands were only observed with wild-type membranes (lane 1) and are denoted by arrows on the left. FP denotes free pigment. The positions of marker proteins and of the TMBZ-stained bands are given in kdaltons.

two alternative interpretations regarding the identity of band C-5. On one hand, it is possible that this band represents heme association with a subunit of cytochrome *b*-559. Certainly, the appearance of this band only following solubilization in the presence of thiols (Fig. 2A) supports this interpretation. Alternatively, it is possible that the band observed in this region represents heme association with the small, 6.6 kdalton subunit of cytochrome b_6. Both of these interpretations are consistent with an extrapolation of the

molecular weight information obtained from isolated cytochromes obtained from both chloroplast and cyanobacterial membranes.

An electrophoretic assessment of mutants which lack spectral evidence of cytochrome activity is an excellent way to identify the TMBZ-oxidizing bands. Several maize mutants have been isolated which possess aberrant cytochrome content [10]. Fig. 5 shows a comparison of the TMBZ-staining pattern in profiles of maize wild-type (lane 1) and mutant *hcf*-6 (lane 2). This mutant lacks both cytochromes *f* and b_6 as determined by oxidized minus reduced difference spectra (Metz and Miles, personal communication). Interestingly, this mutant is also devoid of TMBZ-staining activity at both 34 and 20 kdaltons (Fig. 5). These findings are in agreement with those of Hoyer-Hansen [21] in work on barley polypeptide patterns and emphasize the correlation based solely upon molecular weight estimates. Thus, we conclude that the 33 kdalton TMBZ-stained band (C-2) is cytochrome *f*, whereas the 18.5 kdalton band (C-4) represents a heme-containing subunit of cytochrome b_6.

Thus, we have shown here that cyanobacterial thylakoids contain a series of polypeptides which, following electrophoretic assessment, possess peroxidase activity. That such activity depends upon heme [8] leads directly to the speculation that the banding patterns which we observe correspond to the subunit structure of photosynthetic cytochromes. This contention, as summarized in Table I, is supported both by known molecular weight information on isolated cytochromes and by assessment of mutants possessing aberrant cytochrome content. The TMBZ-staining polypeptides which lie in a range of 13–33 kdaltons are of particular interest in light of the recent findings of Sadewasser and Sherman [1]. Their studies using differential autoradiography showed that most polypeptides between 13 and 20 kdaltons were only slightly accessible to the membrane surface and yet were heavily labeled by lipophilic, photoaffinity probes. An interesting exception to this pattern was a polypeptide at 13.5 kdaltons which was accessible to both lipophilic and hydrophilic labeling reagents. The results of Sadewasser and Sherman [1] suggest an arrangement of polypeptides within the cyanobacterial thylakoid membrane. Integration of the photosynthetic cytochromes into this arrangement would be of great interest. Thus, a major focus of future work will be to utilize the TMBZ-staining procedure to analyze the topography of cytochromes within the photosynthetic membrane.

Acknowledgments

We gratefully acknowledge the assistance of Drs. Donald Miles and James Metz, both in providing the maize material assessed in this report and in many fruitful discussions. Our thanks also extend to Ms. Jill Cunningham for expert technical assistance. This work was supported by NIH grants GM 21827 and GM 07704.

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