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PURIFICATION OF CYTOCHROME *b*-559 FROM OXYGEN-EVOLVING PHOTOSYSTEM II PREPARATIONS OF SPINACH AND MAIZE

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A rapid and simple procedure is presented for the purification of chloroplast cytochrome *b*-559. The method is based on the protocol devised by Garewal and Wasserman (Garewal, H.S. and Wasserman, A.R. (1974) Biochemistry 13, 4063–4071), which we have modified to eliminate the requirement for a lengthy electrophoretic step. Novel features of our method include: the use of oxygen-evolving Photosystem II preparations (Kuwabara, T. and Murata, N. (1982) Plant Cell Physiol. 23, 533–539) as the starting material; isocratic elution of cytochrome *b*-559 from a DEAE-cellulose column (yielding the protein in a pure state); and a simple column procedure for removal of excess Triton X-100. The procedure has been applied to both spinach and maize (*Zea mays* L.). Purified cytochromes *b*-559 from these species have similar optical spectra and mobility during gel electrophoresis under native conditions. Lithium dodecyl sulfate polyacrylamide gel electrophoresis of cytochrome *b*-559 from both spinach and maize reveals a major polypeptide band (apparent molecular mass = 9 kDa), and two minor bands (apparent molecular masses = 10 kDa and 6 kDa).

Introduction

Cytochrome *b*-559 is an integral component of the PS II complex in the chloroplast thylakoid membrane [1]. Although considerable effort has been directed towards determination of its role in PS II, its function remains controversial [2–9]. Analyses of biochemical and biophysical properties of the purified protein could provide new data useful in the search for its function in the photosynthetic process. In 1974, Garewal and Wasserman [10] published a procedure for the purification of cytochrome *b*-559 from spinach thylakoids. The method involved; isolation of chloroplast membranes, removal of the majority of the chlorophyll with ethanol, solubilization of the cytochrome with a Triton X-100/4 M urea solution, passage of the extract through a DEAE column and preparative gel electrophoresis. We report here a modification of their method which eliminates the requirement for the electrophoretic step, thus shortening and simplifying the procedure.

Our protocol has two unique features. First, oxygen-evolving PS II preparations [11] are used as starting material. These can be prepared simply and with high yield and have the advantage of being devoid of cytochromes *f* and *b*-563, proteins which follow cytochrome *b*-559 through many biochemical separation procedures. Secondly, the DEAE-cellulose column was lengthened and used as a chromatographic step. It was found that cytochrome *b*-559 is slightly retained by the column under equilibration conditions, and it can be separated from the chromatographic front. The cytochrome is pure, as judged by optical spectra and

Abbreviations: LDS, lithium dodecyl sulfate; PS, photosystem; TMBZ, 3,3′,5,5′-tetramethylbenzidine; Chl, chlorophyll; Mes, 4-morpholineethanesulfonic acid.

native gel electrophoresis, when it is eluted from the column. Subsequent steps are devoted to removal of excess Triton X-100 and urea and to concentration of the protein.

Analysis of the purified protein using LDS-polyacrylamide gel electrophoresis reveals a major polypeptide band with an apparent molecular mass of 9 kDa. Additionally, two minor polypeptides, with apparent molecular masses of 10 and about 6 kDa are present. The major polypeptide band corresponds to a polypeptide present in thylakoid membranes and PS II preparations.

Cytochrome *b*-559 has also been isolated from maize using this procedure. The maize cytochrome *b*-559 resembles the spinach protein with respect to optical spectra and mobility on native gels. The spinach and maize proteins also have identical polypeptide patterns on LDS-polyacrylamide gels.

Materials and Methods

PS II particles were prepared using the method of Kuwabara and Murata [11] except that 1 mM phenylmethylsulfonyl fluoride was included in all solutions. Spinach was obtained from a local market, maize leaves were harvested from seedlings between 2- and 4-weeks old.

All procedures were performed at 4°C. Buffer solutions were prepared at room temperature. PS II preparations were suspended in 0.3 M sucrose, 10 mM NaCl, 25 mM Mes-NaOH, pH 6.5, 1 mM phenylmethylsulfonyl fluoride [11] at 2 mg Chl/ml and either used immediately or stored at −20°C. Typical isolations started with PS II preparations containing 20–30 mg Chl. The majority of the lipid components of the PS II preparation was removed by extraction with ethanol [12]. 9 vol. of cold, absolute ethanol were added to the suspension and the mixture blended for 30 s in a Sorvall Omni-Mixer. The pellet obtained after centrifugation ($30\,000 \times g_{max}$ for 10 min) was homogenized as before in absolute ethanol (using about one-half the volume of ethanol used in the initial extraction step). Centrifugation yielded a light-brown pellet which was washed with 50 mM Tris-HCl, pH 8.0 (low-speed homogenization in the Omni-Mixer followed by centrifugation at 30 000 $\times g$ for 10 min). The washed pellet was either stored in 40 mM Tris-HCl, pH 8.0, 20% glycerol (w/v) at −20°C or used immediately.

Solubilization of cytochrome *b*-559 was accomplished as described in Ref. 12 with minor modifications. The pellet remaining after ethanol extraction and Tris washing of the PS II preparation was dispersed in 4 M urea, 2% Triton X-100 (w/v), 50 mM Tris-HCl, pH 8.0, 5 mM dithioerythritol and sonicated for 2 min on ice (eight 15-s periods with 1-min cooling intervals using a Model W-375 sonicator, Heat Systems-Ultrasonics, Inc., set at the micro-tip power limit with a 50% duty cycle). Between 1 and 2 ml of solubilization solution were used for every 5 mg of chlorophyll present in the original PS II suspension. The suspension was clarified by centrifugation ($60\,000 \times g_{max}$ for 30 min) and the supernatant loaded on to a DEAE-cellulose column (Whatman DE52). The column, 2.8 × 19 cm, with a bed volume of about 95 ml, was equilibrated with 2 M urea, 2% Triton X-100 (w/v), 50 mM Tris-HCl, pH 8.0, 2 mM dithioerythritol. Sample volume was kept to less than 10% of the bed volume and the column was developed with equilibration solution. The progress of cytochrome *b*-559 can be followed visually. It is observed as a pink band which separates from a more rapidly moving green zone within the first 5–10 cm of the column. Fractions are analyzed spectrophotometrically and those containing cytochrome *b*-559 are pooled.

The majority of Triton X-100 can be removed from the pooled fractions by a second column procedure. A small column (0.7 × 8 cm) of DEAE-Sephadex was prepared and equilibrated with 2 M urea, 50 mM Tris-HCl, pH 8.0, 2 mM dithioerythritol, 0.05% Triton X-100 (w/v). The sample was loaded and the column washed with equilibration solution. Cytochrome *b*-559, which binds to DEAE-Sephadex under these conditions, is then eluted with 50 mM Tris-HCl, pH 8.0, 2 M urea, 2 mM dithioerythritol, 0.05% Triton X-100, 0.2 M NaCl. Urea, salt and additional Triton X-100 are removed by dialysis against 50 mM Tris-HCl, pH 8.0, 2 mM dithioerythritol. The sample was routinely concentrated by the Sephadex G-25 method described by Wasserman [12] or by using an Amicon ultrafiltration apparatus fitted with a UM 30 membrane.

Native gel electrophoresis in the presence of Triton X-100 was performed as described by

Wasserman [12], except that a slab gel apparatus (1.5-mm spacers) was used and the resolving gel had an acrylamide concentration of 9%. Also, thioglycolate was omitted from the tank buffer. Thylakoid membranes from spinach and maize were prepared for LDS-polyacrylamide gel electrophoresis as described by Metz and Miles [13]. Samples of cytochrome *b*-559 were made to 1% LDS and 30 mM dithioerythritol using concentrated stock solutions prior to loading on the gels. Gels were stained for heme-catalyzed peroxidase activity [14] using the method described by Guikema and Sherman [15].

All spectra were obtained with an Aminco DW2 spectrophotometer. Cytochrome concentrations in PS II preparations were determined by chemically induced difference spectra [16] using potassium ferricyanide (60 mM) as an oxidant and a few crystals of sodium dithionite as reductant. For estimation of the ratio of high-potential to low-potential forms of cytochrome *b*-559 potassium ferrocyanide (60 mM) or hydroquinone (200 mM) were used as reductants. Absolute spectra were obtained versus appropriate blanks.

Results

PS II preparations which retain oxygen-evolving capability can be easily prepared from spinach thylakoids [11]. Typically, 20–35% of the chlorophyll present in isolated thylakoids is retained in the final PS II preparation. The preparations were found to be devoid of cytochromes *f* and *b*-563, as judged by difference spectroscopy and by TMBZ/H_2O_2 staining of samples separated on LDS-polyacrylamide gels.

Attempts to isolate PS II preparations from maize were successful only when summer-grown plants were used. When plants grown in a greenhouse in the fall or winter, or plants grown in controlled-environment chambers, were used the PS II yield varied from small to none. No satisfactory explanation for this phenomenon was found. However, if the Triton X-100-to-Chl ratio was lowered to 10:1, active preparations could be obtained from maize. These preparations were contaminated with PS I, but they did not have either cytochrome *f* or *b*-563 and could therefore be used as starting material for the isolation of cytochrome *b*-559.

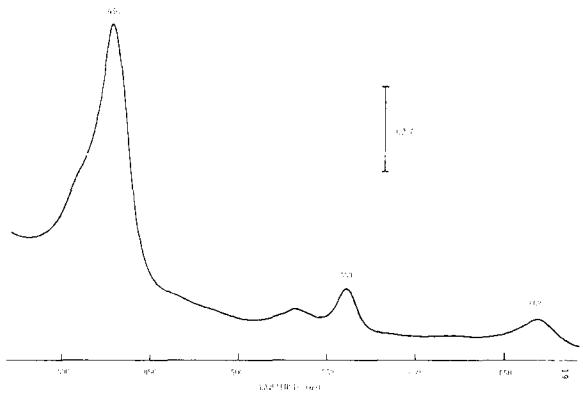

Fig. 1. Absorption spectrum of material solubilized by sonication of ethanol-extracted spinach PS II preparations in the presence of 4 M urea/2% Triton X-100, 5 mM dithioerythritol. The sonicate was clarified by centrifugation prior to obtaining the spectrum.

Analysis of spinach PS II preparations using a dithionite-reduced minus ferricyanide-oxidized difference spectrum indicated a Chl-to-cytochrome *b*-559 ratio of about 200. As judged by reduction with ferrocyanide, approx. 55% of the cytochrome is in the high-potential form.

Ethanol treatment of the particles removes most of the chlorophyll, leaving a pale-brown pellet. Sonication in the presence of 4 M urea and 2% Triton X-100 is used to solubilize the cytochrome. Fig. 1 shows the spectrum of the clarified extract from spinach. Cytochrome *b*-559, reduced by dithioerythritol, is the dominant component of the spectrum. Chl *a* is also present in the spectrum. After solubilization in the urea/Triton X-100 solution all of the cytochrome is in a low-potential form, i.e., not reducible by ferrocyanide or hydroquinone.

The urea/Triton X-100 extract is then applied to a DEAE-cellulose column and the column is developed using starting conditions. Several proteins are bound by the medium and are seen as a yellowish-brown zone at the top of the column. Chl *a*, present in the extract, is observed as a green band which travels with the chromatographic front. This chlorophyll zone can be used to estimate the void volume of the column (approx. 45 ml for the column described in Materials and Methods). Cy-

tochrome *b*-559 is seen as a pink band traveling behind the green front. It is essential not to overload the column to avoid overlapping of the chlorophyll and cytochrome bands. The volume of the applied sample should be equal to or less than 10% of the bed volume. The height-to-diameter ratio of the column should be 6 or greater. In our column, the cytochrome band elutes at about 1.8-times the void volume. Recovery of cytochrome *b*-559 from the column is greater than 90%.

Fig. 2 shows the absorption spectrum of pooled fractions from the DEAE-cellulose column containing isolated cytochrome *b*-559 from maize. The sample was concentrated prior to obtaining the spectrum. Addition of dithionite to the sample of cytochrome does not alter the shape of the spectrum and the absorption peaks but does result in an about 5% increase in the amplitude. Both the absolute spectrum and the difference spectrum (not shown) of the maize protein are very similar to those published for spinach [12].

Native gel electrophoresis of the spinach cytochrome *b*-559 fraction from the DEAE-cellulose column reveals a single protein band (Fig. 3, lane 1). Maize cytochrome *b*-559 also shows a single band with a similar mobility in native gels (Fig. 3, lane 2). The protein, in the native state, has peroxidase activity as measured by the TMBZ/H_2O_2 assay (results not shown).

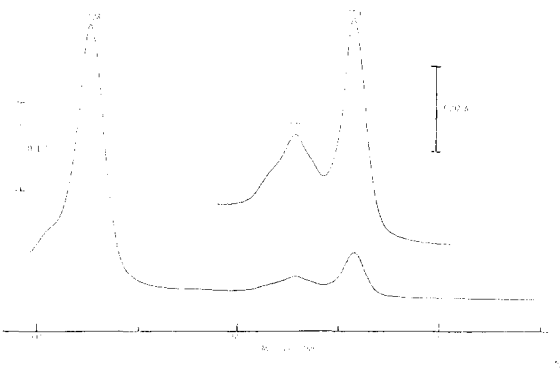

Fig. 2. Absorption spectrum of cytochrome *b*-559 (reduced by dithioerythritol) isolated from maize. Spectrum is of pooled fractions from a DEAE-cellulose column. The sample was concentrated prior to analysis. The upper trace is of the same sample, at higher sensitivity, to show greater detail of the α and β absorption peaks. Reference cuvette contains 50 mM Tris-HCl, pH 8.0, 2 M urea, 2% Triton X-100, 2 mM dithioerythritol.

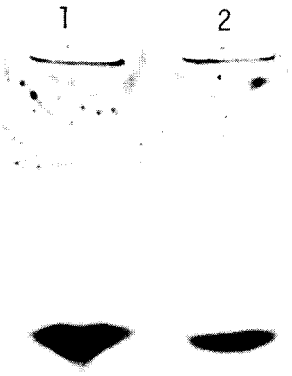

Fig. 3. Native gel electrophoresis of cytochrome *b*-559 isolated from spinach (lane 1) and from maize (lane 2). Samples were taken from the DEAE-cellulose column and were concentrated prior to loading on the gel. A 1.5 mm thick slab gel was used and the proteins revealed by staining with Coomassie brilliant blue.

Attempts to analyze cytochrome fractions, taken directly from the DEAE-cellulose column, on LDS-polyacrylamide gels were unsuccessful. This is due to the presence of 2% Triton X-100 in the sample (the lower portions of the gels showed very poor resolution). We removed the majority of Triton X-100 by binding the protein to DEAE-Sephadex and washing the column with a low Triton X-100 (0.05%, w/v), solution. The protein is then eluted from the column by including 0.2 M NaCl in the wash solution. This step also serves to concentrate the cytochrome. Salt, urea and more Triton X-100 are removed either by dialysis or by several-fold dilutions and reconcentration in an Amicon ultrafiltration cell.

Figs. 4 and 5 show the results of LDS-polyacrylamide gel electrophoresis of the isolated cytochromes, total thylakoid membrane proteins and PS II polypeptides as well as various molecular mass standards. Analysis of purified spinach cytochrome *b*-559 reveals one major and two minor polypeptide bands (Fig. 4B and A, lane 3). One of the minor bands migrates just above the strongly

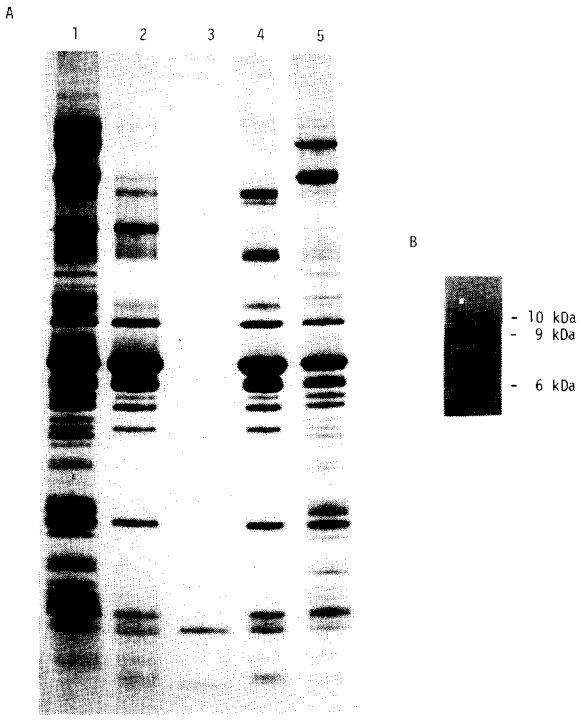

Fig. 4. (A) LDS-polyacrylamide gel electrophoretic analysis of polypeptides of: spinach thylakoid membranes (lane 1), spinach oxygen-evolving PS II preparation (lane 2), isolated spinach cytochrome *b*-559 (lane 3), ethanol-extracted spinach PS II preparation (lane 4), ethanol-extracted spinach thylakoids (lane 5). (B) Spinach cytochrome *b*-559, as in lane 3 of A, shown after a very brief destaining period, to reveal the faint polypeptide band with an apparent molecular mass of 10 kDa.

stained band while the other faint band runs below the major band. The upper band does not stain well with Coomassie brilliant blue, and often cannot be detected after prolonged destaining (e.g., Fig. 4A, lane 3). Fig. 4B shows the polypeptides of spinach cytochrome *b*-559 visible after a brief destaining treatment. This set of polypeptides has been consistently observed in all purified cytochrome *b*-559 samples, including those which have been subjected to native gel electrophoresis prior to separation on LDS gels.

Polypeptide bands with mobilities identical to that of the major band of the purified cytochrome can be observed in samples of spinach thylakoid membranes (Fig. 4A, lane 1) as well as the spinach PS II particles (Fig. 4A, lane 2). This portion of the gel is often obscured by mixed, detergent/lipid

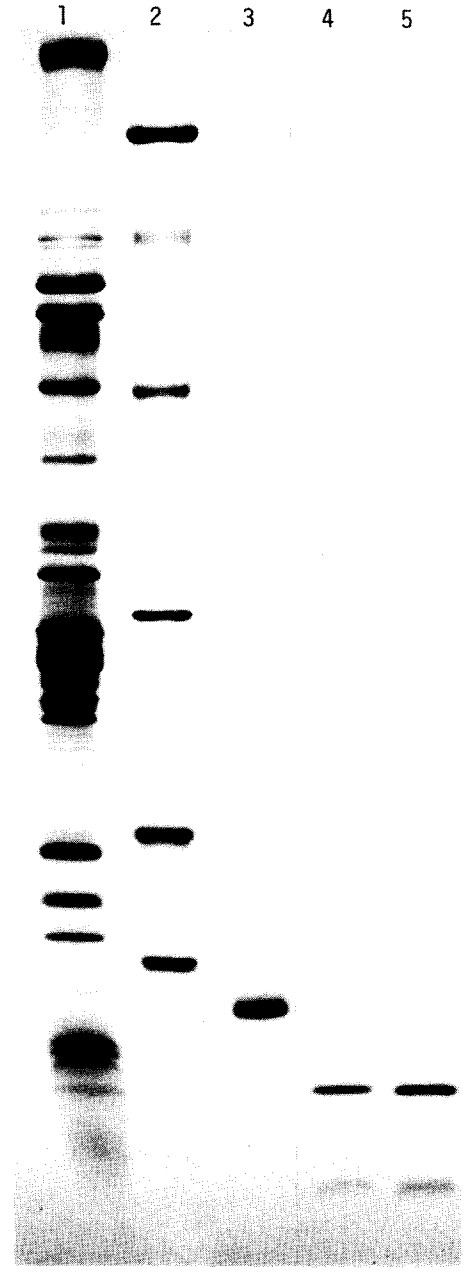

Fig. 5. LDS-polyacrylamide gel electrophoresis analysis of maize thylakoid membranes (the sample was not heated prior to electrophoresis at 4°C) (lane 1); protein molecular mass standards from Bio-Rad Laboratories (phosphorylase *b*, 92500; bovine serum albumin, 66200; ovalbumin, 45000; carbonic anhydrase, 31000; soybean trypsin inhibitor, 21500; and lysozyme, 14400) (lane 2); horse heart cytochrome *c* (12200) (lane 3); purified spinach cytochrome *b*-559 (lane 4); and purified maize cytochrome *b*-559 (lane 5).

micelles when samples contain large amounts of chlorophyll and other lipids. Extraction of the membrane and PS II preparations with ethanol prior to electrophoretic separation, while resulting in the loss of some larger polypeptides from the profile, allows improved resolution in the lower region of the gel (Fig. 4A, lanes 4 and 5). In these samples polypeptides with mobilities similar to those of both the major and lower minor band of the purified cytochrome sample can be observed.

Gel electrophoresis in the presence of anionic detergents is often used to estimate molecular masses of polypeptides [17]. However, this method can be quite unreliable for polypeptides with molecular masses below about 15 kDa, and especially in the case of hydrophobic, membrane proteins [18]. Considering these problems, Fig. 5 shows the molecular mass standards we have used to estimate the apparent molecular masses of the polypeptides of cytochrome *b*-559. Lane 2 contains standards from Bio-Rad Laboratories (see figure legend) and lane 3 contains a sample of horse heart cytochrome *c* (12.2 kDa). By extending the curve obtained with these standards, we found values of 10 kDa for the upper minor polypeptide, 9 kDa for the major band and about 6 kDa for the lowest band.

Lanes 4 and 5 of Fig. 5 contain purified samples of cytochrome *b*-559 isolated from spinach and maize, respectively. No differences in the polypeptide patterns could be detected between these samples. A polypeptide with a mobility similar to that of the major cytochrome band can be observed in maize thylakoids (lane 1).

Heme-catalyzed peroxidase activity measured by the TMBZ/H_2O_2 procedure [14,15] has been used to identify cytochromes *f* and *b*-563 in LDS-polyacrylamide gels. With our electrophoretic conditions, we have found no peroxidase-staining bands associated with the purified cytochrome *b*-559 samples under denaturing conditions.

Discussion

We have presented a rapid and reliable procedure for the isolation of cytochrome *b*-559. The method differs from those of Garewal and Wasserman [10] and Zielinski and Price [19] in that it avoids the requirement for a lengthy native gel electrophoretic step. It also differs from a recent procedure, used to isolate cytochrome *b*-559 from barley [20], in the specific chromatographic techniques employed and that our procedure utilizes PS II preparations as the starting material. The latter is a crucial point, since the PS II preparations can be prepared with high yield and are devoid of cytochromes *f* and *b*-563.

Analysis of both spinach and maize cytochrome *b*-559 using LDS-polyacrylamide gel electrophoresis consistently reveals three low molecular mass polypeptides. One of the polypeptides (apparent molecular mass = 9 kDa) stains much more intensely than the other two. Garewal and Wasserman [21] reported the presence of three different kinds of polypeptide chains, based on N-terminal analysis, in their cytochrome *b*-559 preparation. However, they observed only one size of polypeptide chain, with an apparent molecular mass of about 6 kDa. This polypeptide is probably the same as the major band which we observe. The other two bands could be easily overlooked due to their relatively poor staining with Coomassie brilliant blue. The difference in molecular mass values can readily be accounted for by use of different marker proteins. Koenig and Møller [20] reported that cytochrome *b*-559, isolated from barley, has a major polypeptide (molecular mass = 9.3 kDa) and a minor component (molecular mass = 9 kDa). These are likely to correspond to the 9 and 6 kDa polypeptides which we observe in spinach and maize. In addition, their preparation contained two components (molecular mass 32 and 30 kDa) which did not stain with Coomassie brilliant blue, but could be visualized by TMBZ/H_2O_2 staining. These TMBZ/H_2O_2 staining bands were observed only in freshly prepared samples, and were heat sensitive. Koenig and Moller rejected the possibility of cytochrome *f* contamination as a source for these bands, based primarily on their heat sensitivity. In contrast, we have not found any TMBZ/H_2O_2 staining bands in our preparations. Lach and Boger [22] reported a subunit molecular mass of 37 kDa for spinach cytochrome *b*-559. We have not observed any polypeptides in this region with our samples.

Elucidation of the native molecular properties of cytochrome *b*-559 may be essential to the interpretation of its function. Several of the properties

which have been reported need to be reexamined. For example, Garewal and Wasserman [21] presented evidence that the native protein has a lipid complement which includes Chl *a* and β-carotene. As indicated by those investigators, this is clearly at odds with the spectral data. Additionally, it has been estimated that the native protein contains eight polypeptide subunits [21]. This was based on a value of 45 900 g protein/mol heme and an estimated value of 5.6 kDa for the polypeptide subunits. However, one cannot rely on SDS-polyacrylamide gel electrophoresis to provide accurate determinations of molecular masses for proteins which are this small. Koenig and Møller [20] have pointed out that, based on amino acid composition, the lowest possible molecular mass for the major polypeptide of cytochrome *b*-559 is 9 kDa. Also, a different value for the protein/heme ratio has been reported [20]. The question of the polypeptide and lipid organization of this protein is clearly unresolved. The availability of simplified isolation procedures has allowed purification of milligram quantities of cytochrome *b*-559 and will aid in these studies.

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References

1 Boardman, N.K. and Anderson, J.M. (1967) Biochim. Biophys. Acta 143, 187–203
2 Butler, W.L. (1978) FEBS Lett. 95, 19–25
3 Cramer, W.A. and Whitmarsh, J. (1977) Annu. Rev. Plant Physiol. 28, 133–172
4 Bendall, D.S. (1982) Biochim. Biophys. Acta 683, 119–151
5 Henningsen, K.W. and Boardman, N.K. (1973) Plant Physiol. 51, 1117–1126
6 Horton, P., Whitmarsh, J. and Cramer, W.A. (1976) Arch. Biochem. Biophys. 176, 519–524
7 Cramer, W.A., Whitmarsh, J. and Horton, P. (1979) in The Porphyrins (Dolphin, D., ed.), Vol. 7, pp. 71–106, Academic Press, New York
8 Heber, U., Kirk, M.R. and Boardman, N.K. (1976) Biochim. Biophys. Acta 546, 292–306
9 Whitmarsh, J. and Cramer, W.A. (1977) Biochim. Biophys. Acta 460, 280–289
10 Garewal, H.S. and Wasserman, A.R. (1974) Biochemistry 13, 4063–4071
11 Kuwabara, T. and Murata, N. (1982) Plant Cell Physiol. 23, 533–539
12 Wasserman, A.R. (1980) Methods Enzymol. 69, 181–202
13 Metz, J.G. and Miles, D. (1982) Biochim. Biophys. Acta 681, 95–102
14 Thomas, P.E., Ryan, D. and Levine, W. (1976) Anal. Biochem. 75, 168–176
15 Guikema, J.A. and Sherman, L.A. (1981) Biochim. Biophys. Acta 637, 189–201
16 Bendall, D.S., Davenport, G.E. and Hill, R. (1971) Methods Enzymol. 23, 327–344
17 Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406–4412
18 Tanford, C. and Reynolds, J.A. (1976) Biochim. Biophys. Acta 457, 133–170
19 Zielinski, R.E. and Price, C.A. (1982) in Methods in Chloroplast Molecular Biology (Edelman, M., Hallick, R.B. and Chua, N.-H., eds.), pp. 933–944, Elsevier, Amsterdam
20 Koenig, F. and Møller, B.L. (1982) Carlsberg Res. Commun. 47, 245–262
21 Garewal, H.S. and Wasserman, A.R. (1974) Biochemistry 13, 4072–4079
22 Lach, H.-J. and Böger, P. (1976) Z. Naturforsch. 32c, 75–77