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A 4-kDa maize chloroplast polypeptide associated with the cytochrome b_6–f complex: Subunit 5, encoded by the chloroplast *petE* gene

(chloroplast genes/electron transport)

JEAN HALEY AND LAWRENCE BOGORAD

Department of Cellular and Developmental Biology, Harvard University, Cambridge, MA 02138

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ABSTRACT **Four polypeptides, three of which are chloroplast-encoded, have been shown to be associated with the thylakoid membrane cytochrome b_6–f complex. In this report, the gene for a fifth polypeptide, which copurifies with the b_6–f complex, is identified through the use of an antibody generated against a synthetic decapeptide predicted from a maize chloroplast DNA sequence. The deduced 37-amino acid sequence of the immunoreactive 4-kDa polypeptide is 100% and 86% conserved in the respective similar open reading frames encoded by *Nicotiana tabacum* and *Marchantia* chloroplast DNA. The 4-kDa polypeptide is present in both etioplasts and chloroplasts of maize and is found as well in spinach, tobacco, pea, wheat, and rice thylakoids. Similar to the other subunits of the b_6–f complex, it is intrinsic to the membrane, and its hydrophilic COOH terminus is located at the stromal thylakoid surface. We propose to call the 4-kDa polypeptide "subunit 5" and the chloroplast gene that encodes it the *petE* gene.**

The cytochrome b_6–f complex is located in the thylakoid membranes of plants, algae, and cyanobacteria and functions in the linear cross-membrane transport of electrons between photosystem II and photosystem I, as well as in cyclic electron flow around photosystem I (reviewed in ref. 1). Although the b_6–f complex has also been implicated in the transport of protons across the thylakoid membrane, the mechanisms for accomplishing and regulating electron transport-linked proton translocation are not as yet understood and remain a focus of research (1–3).

Four polypeptides associated with the purified b_6–f complex, as well as the chloroplast and nuclear genes that encode them, have been characterized. The spinach b_6–f complex includes a heterogeneous 33- to 34-kDa cytochrome *f* polypeptide carrying one *c*-type heme, a 23-kDa cytochrome b_6 polypeptide carrying two *b*-type hemes, a 20-kDa Fe-S Rieske protein, and a 17-kDa polypeptide, which has been called subunit 4 (4). Similar components are found in preparations of the complex from other organisms, but the apparent molecular masses are variable (1, 5–7). The Rieske protein is nuclear-encoded, but the other subunits are products of the chloroplast *petA* (cytochrome *f*), *petB* (cytochrome b_6), and *petD* (subunit 4) genes (8–10). Several small polypeptides of apparent molecular mass <10 kDa have been consistently detected in preparations of the b_6–f complex (4, 5, 7) but have not been further characterized as subunits of the complex.

We report here that a polypeptide migrating with an apparent molecular mass of 4 kDa in NaDodSO$_4$/polyacrylamide gels of a spinach cytochrome b_6–f preparation is the product of a chloroplast gene. The 4-kDa polypeptide is recognized by an antiserum generated against a decapeptide representing the deduced COOH terminus of a 37-amino acid reading frame on the maize chloroplast genome. Immunoblot analyses of thylakoid membrane fractions and a b_6–f complex preparation indicate that the 4-kDa polypeptide is uniquely associated with polypeptides of the b_6–f complex. In accordance with current nomenclature, we propose to name the 4-kDa polypeptide "subunit 5" and the chloroplast gene that encodes it the *petE* gene.*

METHODS

DNA Sequence and Analysis. M13mp18 and M13mp19 clones with maize chloroplast DNA inserts derived from plasmid pZmc503 containing *Bam*HI fragment 15′ (11) were used as single-stranded templates for sequencing (12). Sequences were analyzed with the Pustell DNA/Protein Sequence Analysis program (IBI).

Chloroplast Preparations and Thylakoid Fractionations. Chloroplasts and thylakoid membranes were isolated from seedling leaves of *Zea mays* (FR9 cms × FR37) (Illinois Foundation Seed) and leaves of market spinach (13). Spinach thylakoid membrane complexes were fractionated according to Steinback *et al.* (14). The spinach cytochrome b_6–f complex was prepared by extraction of thylakoids with octyl glucoside cholate (15).

Electrophoresis and Immunoblotting. Polypeptides were analyzed by NaDodSO$_4$/PAGE on either 12–18% gradient gels (16) or 20% gels adapted for the resolution of small polypeptides (17). For immunoblot analyses, gels were blotted onto Immobilon polyvinylidene difluoride membrane (Millipore) (18), and reactions were visualized with alkaline phosphatase-linked goat anti-rabbit secondary antibody (Promega).

Preparation of Synthetic Peptide Antiserum. A decapeptide (Gln-Tyr-Arg-Arg-Gly-Asp-Gln-Leu-Asp-Leu) (Fig. 1 *A* and *C*) was synthesized and coupled to keyhole limpet hemocyanin with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride before injection into rabbits (19). Enzyme-linked immunosorbent assay and competition immunoblot analysis, using synthetic peptide as antigen and competitor, respectively, were used to determine the reactivity and specificity of the antiserum.

Proteolysis of Thylakoid Membranes. Intact thylakoids were incubated for 1 hr on ice in 1 mM Tricine, pH 7.4/100 mM sucrose to unstack membranes before treatment with either trypsin or carboxypeptidase A phenylmethylsulfonyl fluoride (bovine pancreas, type I PMSF; Sigma C-6393).

RESULTS

*Bam*HI fragment 15′ of the maize chloroplast chromosome (11) contains several small open reading frames (ORFs) that

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Abbreviations: ORF, open reading frame; CF, chloroplast coupling factor.

*The sequence reported in this paper is being deposited in the EMBL/GenBank data base (accession no. J04502).

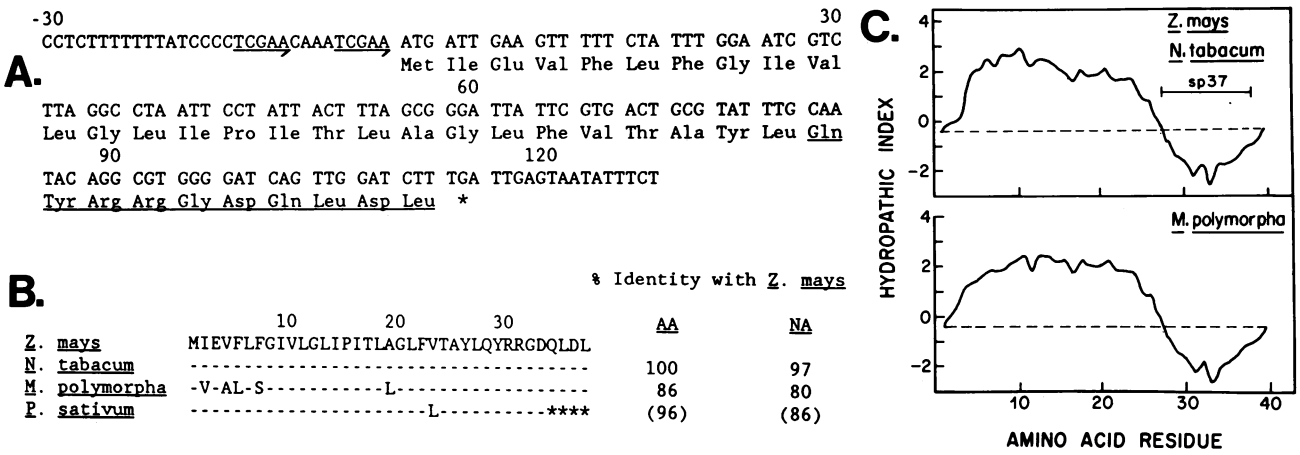

FIG. 1. (*A*) Nucleotide sequence and deduced amino acid sequence of the maize chloroplast gene encoding the 4-kDa polypeptide. Sequence shown represents a region of *Bam*HI fragment 15′ (coordinates 44401–44272) (11). The underlined COOH-terminal 10 amino acid sequence corresponds to that of the synthetic peptide (sp37) used to raise antibody. (*B*) Comparison of derived amino acid sequences (single-letter code) from similar maize, tobacco, *Marchantia*, and pea reading frames. The tobacco sequence is derived from the "A" strand (nucleotides 68562–68675 in ref. 9); *Marchantia* sequence is derived from ORF 37 on "+" strand (nucleotides 64370–64483 in ref. 10); pea sequence is derived from nucleotide sequence C (positions 693–595 in ref. 20). Asterisks at 3′ end of pea sequence denote sequence divergence (expression of the 4-kDa polypeptide in pea thylakoids suggests that the divergence represents sequencing error). (*C*) Hydropathy profiles of the derived maize, tobacco, and *Marchantia* 4-kDa polypeptides (21). Bar labeled sp37 indicates domain represented by the sequence of the synthetic decapeptide antigen. AA, amino acid sequence; NA, nucleic acid sequence.

are highly conserved in both tobacco and *Marchantia* chloroplast genomes (9, 10) and are encoded on a complex family of transcripts that accumulate in both light- and dark-grown seedling leaves (unpublished data). One of these ORFs specifies a largely hydrophobic 37-amino acid polypeptide (Fig. 1) with a calculated molecular weight of 4152 (Pustell DNA Sequence Analysis, IBI). The deduced amino acid sequence of the maize ORF is identical to the deduced tobacco sequence and differs from the *Marchantia* sequence in only five residues, four of which are conservative replacements in the NH_2-terminal region of the hydrophobic domain (Fig. 1*B*). The ORF is conserved in its position in the three chloroplast chromosomes and is found ≈250 base pairs downstream and on the strand opposite to the *trnW* gene (9, 10, 22). A pea sequence encoding 32 of the first 33 amino acids of the maize and tobacco sequences (Fig. 1*B*) is located in similar proximity to the *trnW* gene, which is situated 790 base pairs upstream and on the opposite strand of the *psbB-psbH-petB-petD* gene cluster in the pea chromosome (20). The conservation of this ORF, as well as the accumulation of complementary transcripts (data not shown), indicated that it might encode a functional polypeptide. A synthetic decapeptide (sp37) representing the deduced hydrophilic COOH-terminal sequence of the ORF (Fig. 1 *A* and *C*) was used to raise antiserum for immunoblot analysis of chloroplast polypeptides.

Immunoreaction of the Synthetic Peptide Antiserum with a 4-kDa Thylakoid Polypeptide. The anti-sp37 antiserum recognizes a single maize thylakoid polypeptide with an apparent molecular mass of 4 kDa (Fig. 2*A*). This apparent size corresponds closely to the calculated size (M_r, 4152) of the deduced 37-amino acid sequence. The immunoreactive thylakoid polypeptide is present in etioplasts of dark-grown maize seedling leaves but accumulates to greater levels within greening plastids as etiolated leaves are exposed to light (Fig. 2*B*). The 4-kDa polypeptide is also present in thylakoids of spinach, tobacco, pea, wheat, and rice (data not shown), indicating that it is widely expressed among higher plants in both monocot and dicot species; however, no polypeptides reacting with the anti-sp37 antiserum were detected in the membranes of the green alga *Chlamydomonas reinhardtii* or the cyanobacterium *Synechocystis* 6803.

Localization of the 4-kDa Polypeptide: Fractionation of Thylakoid Membrane Complexes. To localize the 4-kDa polypeptide within the thylakoid membrane system of protein complexes, components of spinach chloroplast membranes were solubilized with the detergent Triton X-100 and separated on a sucrose gradient (14), and the synthetic peptide antiserum was used to assay gradient fractions for the 4-kDa polypeptide. Fig. 3 shows the distribution of membrane protein complexes in the gradient as identified by their Coomassie blue-stained (Fig. 3*A*) and/or immunoblotted (Fig. 3*B*) constituent marker polypeptides. The method achieves a good partial separation of the complexes, which have overlapping distributions but discrete peaks and characteristic patterns of migration. As described previously (14), the light-harvesting complex is found in the upper third, the cytochrome b_6-f and photosystem II complexes are in the middle, the CF_1-CF_0-ATPase (CF, chloroplast coupling factor) complex is in the lower half, and the self-aggregated photosystem I complex is at the bottom of the gradient.

Immunoblot analysis of the gradient fractions using the anti-sp37 antiserum shows that the 4-kDa polypeptide migrates in the upper-middle region of the gradient, with a distribution that coincides with that of the cytochrome *f*

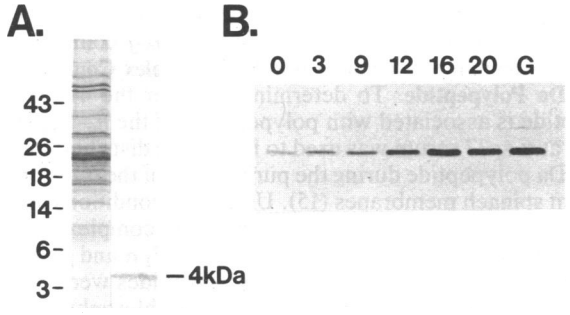

FIG. 2. Immunoreaction of anti-sp37 antiserum with a 4-kDa thylakoid polypeptide. (*A*) Polypeptides of maize chloroplast membranes (5 μg of chlorophyll) were separated on a $NaDodSO_4$/20% polyacrylamide gel (17) and either Coomassie blue-stained (left lane) or analyzed via immunoblotting for reaction with the anti-sp37 antiserum (right lane). (*B*) Immunoblot analysis of the abundance of the 4-kDa polypeptide in plastids of dark-grown maize leaves greened for 0, 3, 9, 12, 16, or 20 hr or in plastids of light-grown leaves (lane G).

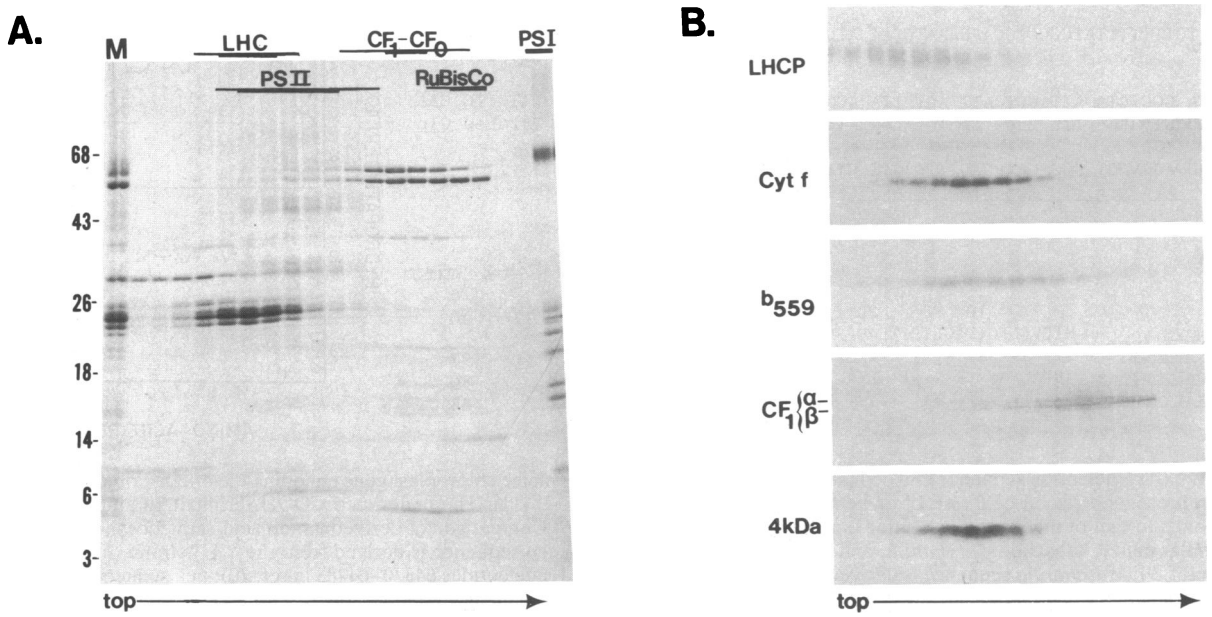

FIG. 3. Sucrose density gradient fractionation of Triton X-100-solubilized thylakoid membrane complexes. Unstacked spinach thylakoids were incubated with Triton X-100 under gentle conditions, which released 95% of the chlorophyll from the membrane; soluble components were subsequently fractionated on a 0.1–0.7 M sucrose gradient containing 0.05% Triton X-100 (14). (*A*) Coomassie blue-stained $NaDodSO_4$/12–18% polyacrylamide gradient gel showing polypeptides in selected gradient fractions; lane M shows solubilized membranes applied to the gradient. Bars above the gradient mark peak fractions of light-harvesting complex (LHC), photosystem II (PSII), CF_1-CF_0 ATPase, photosystem I (PSI), and residual ribulose-bisphosphate carboxylase (RuBisCo) associated with the membranes. (*B*) Immunoblot showing the distribution in the gradient of the 4-kDa polypeptide and components of major membrane complexes. Gradient fractions in *A* were analyzed. LHCP, light-harvesting chlorophyll protein; Cyt f, cytochrome *f* polypeptide; b_{559}, cytochrome b_{559} 9-kDa α polypeptide.

marker polypeptide of the cytochrome b_6–*f* complex (Fig. 3*B*). None of the immunoreactive polypeptide is present in the soluble fraction at the top of the gradient; its position in the gradient implies that it migrates as a member of a complex of polypeptides. A 4-kDa polypeptide is clearly not associated with fractions enriched in ATPase and photosystem I complexes, in the lower half (Fig. 3 *A* and *B*) and the bottom of the gradient (Fig. 3*A*), respectively. The cytochrome b_6–*f* and photosystem II complexes are not well-separated on this gradient, but the distribution of the 4-kDa polypeptide more closely resembles that of the b_6–*f* cytochrome *f* polypeptide than of the photosystem II 9-kDa cytochrome b_{559} polypeptide (Fig. 3*B*).

The coincident distribution of the 4-kDa and cytochrome *f* polypeptides in the gradient is compatible with their being components of the same functional membrane complex. However, this fractionation does not clearly rule out an association of the 4-kDa polypeptide with either photosystem II or another complex comigrating with b_6–*f* components.

Preparation of a Cytochrome b_6–*f* Complex Containing the 4-kDa Polypeptide. To determine whether the 4-kDa polypeptide is associated with polypeptides of the b_6–*f* complex, the anti-sp37 serum was used to follow the distribution of the 4-kDa polypeptide during the purification of the b_6–*f* complex from spinach membranes (15). Under the conditions used for octyl glucoside cholate extraction of the complex from the membranes, significant amounts of the CF_1 α and β subunits and the 4-kDa and cytochrome *f* polypeptides were released into the soluble fraction (Fig. 4*A*). No chlorophyll and no detectable amounts of two identified photosystem II components were released; the 9-kDa cytochrome b_{559} and 44-kDa chlorophyll *a*-binding polypeptides of photosystem II were present in the extracted membranes but not in the soluble fraction containing the CF_1, cytochrome *f*, and 4-kDa polypeptides (Fig. 4*A*). The soluble membrane components were further fractionated by ammonium sulfate precipitation (Fig. 4*B*). The 4-kDa and the cytochrome *f* polypeptides have the same fractionation profile and are most abundant in the 55–65% ammonium sulfate fraction (Fig. 4*B*, lane 3). The two prominent polypeptides in this fraction are identified as cytochrome *f* and cytochrome b_6 by immunoblot (*f*, Fig. 4*C*, lane 3) and heme-staining (23) (*f* and b_6, Fig. 4*C*, lane 1). The immunoreactive 4-kDa polypeptide aligns with the larger of two small Coomassie blue-stained polypeptides that peak in this fraction (Fig. 4*C*, lane 3).

The 55–65% ammonium sulfate fraction was separated on a sucrose gradient (15) and the gradient was analyzed by $NaDodSO_4$/PAGE (Fig. 5*A*). Fractions 10–16 represent the brown zone in the gradient containing the heme-carrying polypeptides of the b_6–*f* complex; they are enriched in four polypeptides that migrate in the gel at the expected positions for the four identified polypeptides of the b_6–*f* complex (4) (marked by arrowheads at right of gel, Fig. 5*A*). Several small polypeptides in the 3- to 6-kDa size range are also enriched in the same fractions and peak together with the larger polypeptides in fractions 12 and 13. The cytochrome *f* and 4-kDa polypeptides have identical distributions in the gradient (Fig. 5*B*). The series of fractions analyzed by immunoblot in Fig. 5*B* shows the concentration of both polypeptides peaking in fraction 13. In addition to the four large polypeptides in the peak fraction 13 of the b_6–*f* preparation, there are four small polypeptides with apparent molecular masses of 3.2, 3.6, 4, and 6 kDa (Fig. 5*C*, lane 2); the anti-sp37 antiserum reacts with a polypeptide that aligns with the 4-kDa polypeptide (lane 3). The cytochromes *f* and b_6 are identified by heme-staining (23) (lane 1), and the cytochrome *f* polypeptide is also identified by immunoblotting (lane 3). The two other abundant polypeptides in this fraction (lane 2) migrate in the gel at appropriate positions for two other characterized components of the complex, the 20-kDa Rieske and the 17-kDa subunit 4 polypeptides (4). These results indicate that all of the 4-kDa polypeptide released by detergent extraction of the thylakoid membrane copurifies with components of the cytochrome b_6–*f* complex.

The 4-kDa Polypeptide Is Integral to the Membrane. The 4-kDa polypeptide has the characteristics of an intrinsic

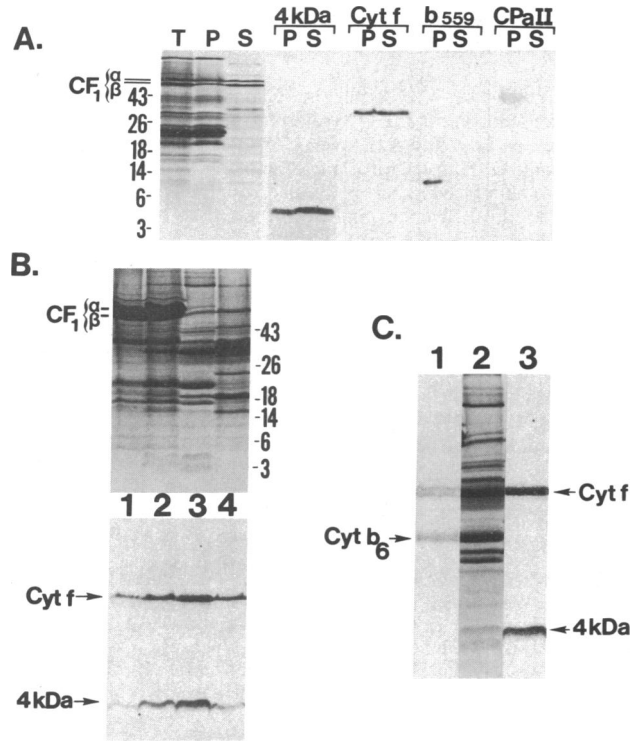

FIG. 4. Octyl glucoside/cholate extraction of thylakoid membranes. Spinach thylakoids were extracted with 0.4% cholate/25 mM octyl glucoside (15). (*A*) Coomassie blue-stained $NaDodSO_4$ gel (17) (*Left*) shows total membranes (8 μg of chlorophyll) before detergent extraction (lane T); membrane pellet after extraction (lane P); and soluble fraction (lane S). Immunoblots (*Right*) show distribution of the 4-kDa cytochrome *f*, 9-kDa cytochrome b_{559}, and 44-kDa chlorophyll *a*-binding photosystem II (CPaII) polypeptides in membrane pellet (lanes P) and soluble fraction (lanes S). (*B*) Ammonium sulfate fractionation of soluble membrane components. Coomassie blue-stained $NaDodSO_4$ gel (17) (*Upper*) shows polypeptides present in four ammonium sulfate fractions: lane 1, 35–45%; lane 2, 45–55%; lane 3, 55–65%; lane 4, 65–95%. Immunoblot (*Lower*) shows the distribution of the cytochrome *f* and 4-kDa polypeptides. (*C*) Identification of b_6–*f* polypeptides in the 55–65% ammonium sulfate fraction. Lane 1, heme-stained gel (23); lane 2, Coomassie blue-stained gel; lane 3, immunoblot incubated with anti-cytochrome *f* and anti-sp37 sera.

membrane component. Extractions of membranes with buffers that remove peripheral membrane polypeptides and complexes (e.g., 1 mM EDTA, 1.0 M Tris) fail to remove it from the membrane; like cytochrome *f* and other membrane-spanning polypeptides, it is not removed from the thylakoid membrane by extraction with 0.1 M NaOH (26) (data not shown). Hydropathy plot analysis of the polypeptide sequence predicts a hydrophobic domain of 27 residues, which is of sufficient length to span the membrane bilayer once as an α-helix (Fig. 1*C*). The algorithm of Goldman and coworkers (FOAMPC program) (27) further identifies this region as one that is likely to form a transbilayer helix.

Membrane Topology of the 4-kDa Polypeptide. The anti-sp37 antiserum, which recognizes the COOH-terminal sequence of the 4-kDa polypeptide, no longer reacts with it in unstacked spinach and maize thylakoids treated with carboxypeptidase A (Fig. 6, lanes C). This implies that the hydrophilic COOH terminus of the 4-kDa polypeptide is located at the exposed stromal surface of the membrane, where it is sensitive to carboxypeptidase digestion. The effects of protease treatment on two embedded polypeptides of known orientation in the membrane, the cytochrome *f* and 9-kDa b_{559}–α polypeptides, attest to the intactness and stromal orientation of the treated membranes. The COOH terminus of the cytochrome *f* polypeptide is located at the stromal membrane surface (28, 29), whereas the NH_2 and COOH termini of the b_{559} polypeptide are located at the stromal and lumenal surfaces, respectively (30). Treatment of intact (or right-side-out) thylakoids with carboxypeptidase, but not with trypsin, removes a 1-kDa region of the cytochrome *f* polypeptide (ref. 28; Fig. 6, lanes C). Conversely, trypsin, but not carboxypeptidase, digests a 1-kDa region of the b_{559} polypeptide at the stromal surface (as shown in Fig. 6, lanes T); the truncated and the full-length b_{559} polypeptide (lanes T and C, respectively) react with antiserum specific for the COOH-terminal sequence at the lumenal surface, indicating that polypeptide sequences located at this surface are not exposed to protease digestion.

DISCUSSION

In this report, we identify a chloroplast gene that encodes a 4-kDa thylakoid membrane polypeptide associated with components of the cytochrome b_6–*f* complex prepared from spinach membranes. The generation of a synthetic peptide antiserum that specifically recognized the 4-kDa polypeptide permitted the sensitive detection of the polypeptide in complex mixtures of membrane components. Using immunoblot

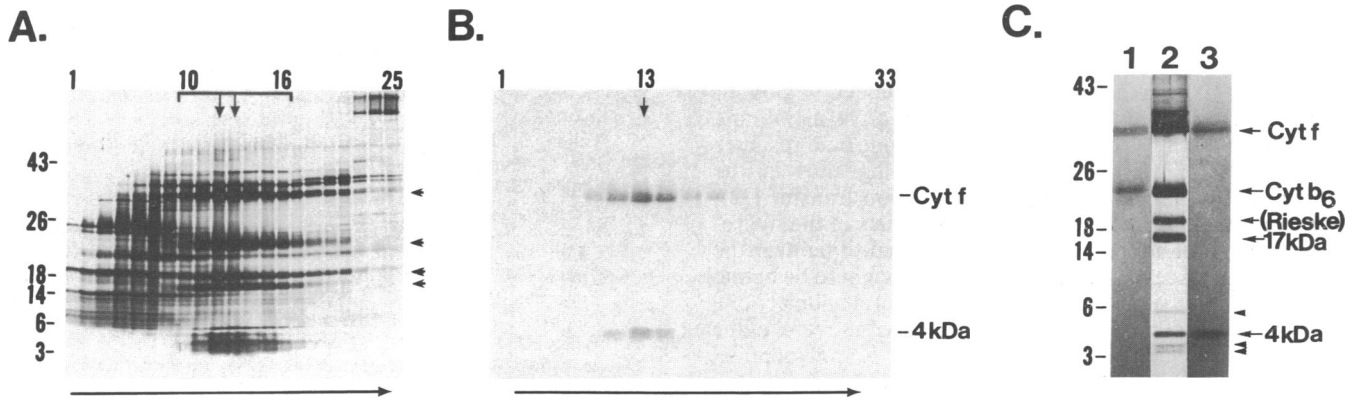

FIG. 5. Association of the 4-kDa polypeptide with the spinach cytochrome b_6–*f* complex. Polypeptides in the 55–65% ammonium sulfate fraction (Fig. 4) were separated on a sucrose density gradient containing 0.05% Triton X-100 (22). (*A*) Silver-stained (24) $NaDodSO_4$ 12–18% gradient gel showing polypeptides in fractions 1, 4–20, and 23–25 of the gradient. Fractions 10–16 represent brown zone of gradient; arrows mark b_6–*f* peak. Arrowheads at right mark migration of b_6–*f* polypeptides. Numbers on left are kDa. (*B*) Immunoblot of alternate gradient fractions 1–33 separated on a 20% $NaDodSO_4$ gel; blot was incubated with anti-cytochrome *f* and anti-sp37 sera. (*C*) Gradient fraction 13 was precipitated with trichloroacetic acid to remove Triton X-100, resuspended without heating, and separated on a long (20 cm) 20% $NaDodSO_4$ gel (17) to resolve low molecular weight polypeptides. Lanes: 1, heme-stained gel (23); 2, silver-stained gel (25); 3, immunoblot incubated with anti-cytochrome *f* and anti-sp37 sera. Numbers on left are kDa.

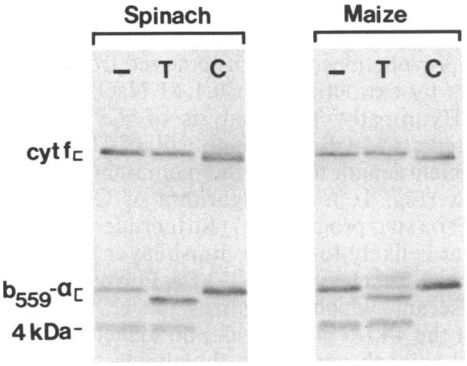

FIG. 6. Effect of protease treatment on the 4-kDa polypeptide in intact spinach and maize thylakoid membranes. Unstacked thylakoids were suspended in 10 mM Tris·HCl (pH 7.8) at 300 μg of chlorophyll per ml and incubated at 20°C for 60 min with either trypsin or carboxypeptidase A phenylmethylsulfonyl fluoride at 50 μg/ml. Membrane samples (5 μg of chlorophyll) were acetone-extracted and used for immunoblot analysis. Blots were incubated with anti-sp37, anti-cytochrome *f*, and anti-b_{559} sera. The anti-b_{559} serum was a synthetic peptide antiserum generated against the COOH terminus of the 9-kDa α polypeptide (gift of W. A. Cramer, Purdue University). Lanes represent untreated membranes (lanes –), trypsinized membranes (lanes T), and membranes treated with carboxypeptidase A (lanes C).

analysis, we have shown that in the course of detergent fractionations of thylakoid membranes and the preparation of a b_6-*f* complex, the 4-kDa polypeptide is consistently enriched in parallel with b_6-*f* polypeptides. The anti-sp37 antiserum did not recognize any small polypeptide present in spinach or maize photosystem II preparations (data not shown). The reactive polypeptide was present, however, in maize stroma lamellae (24) containing b_6-*f* polypeptides but depleted in photosystem II, and in a preparation of spinach b_6-*f* complex provided to us by G. Hauska (Universität Regensberg) (data not shown). Although we have no information regarding the functional role of the 4-kDa polypeptide in the b_6-*f* complex, its coenrichment with the four authentic b_6-*f* subunits strongly implicates it as a fifth member of the complex. We therefore propose to call the 4-kDa polypeptide "subunit 5." Following the current alphabetical nomenclature (31), we also propose to name the chloroplast gene that encodes the 4-kDa subunit 5 polypeptide the *petE* gene.

Much about the structure and function of the cytochrome b_6-*f* complex remains unknown or controversial (1–3). Functional sites on the membrane-spanning regions of b_6-*f* polypeptides involved in the cross-membrane transfer of electrons and protons are largely unidentified. Two structural features of the predicted membrane-embedded region of subunit 5 may be of interest with regard to cross-membrane functions of the b_6-*f* complex. Aromatic amino acid residues have been implicated as possible liganding sites in the membrane for quinones involved in electron transfer (32). There are phenylalanine and tyrosine residues in the hydrophobic region of subunit 5 that are predicted to be near the membrane surfaces, where quinones are thought to be bound (1–3). Secondly, subunit 5 shares the structural anomaly of a membrane-embedded proline (Pro-15) (33) with several of the hydrophobic regions of the cytochrome b_6 polypeptide; others have speculated on the role of embedded prolines in conformational changes linked to cross-membrane proton transport (34). The COOH terminus of subunit 5 is located at the stromal membrane surface, an orientation shared with all four previously characterized b_6-*f* polypeptides (28, 29). A role for the subunit 5 polypeptide might be established by determining the effect(s) of anti-sp37 serum binding to the COOH terminus on functions of the complex—such as proton pumping—assayed in intact membranes or in vesicles containing a reconstituted complex.

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