Electrostatic destabilization of the cytochrome b_6f complex in the thylakoid membrane

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Three of the membrane-spanning polypeptides of the chloroplast cytochrome (cyt) b_6f complex were sequentially released from the thylakoid membrane, in the order cyt b_6, suIV and Rieske iron-sulfur protein, as the pH was increased from 10 to 12, a protocol usually employed to remove peripheral proteins from membranes. The fourth polypeptide of the cyt b_6f complex, cyt f, which spans the membrane once, was apparently not released. The pH values for half-release at low ionic strength were ~10.7, 11.1 and 11.3 respectively. The separation of the polypeptides of the complex and the sequential release is readily seen at pH 11, where the loss from the membrane of cyt b_6, suIV and Fe iron-sulfur center is ~90%, 50% and 20%, respectively. The release of cyt b_6 from the membrane was reflected by the absence of its characteristic reduced minus oxidized absorbance signal. The pH values at which the release occurred increased as the ionic strength was raised, implying that the release of the b_6f polypeptides arises from extrusion due to repulsive electrostatic interactions probably caused by deprotonation of tyrosine and lysine residues. The lipid content of the released polypeptides was very low, consistent with the observation of a non-membranous state. It is proposed that the pH-dependent extrusion requires two electrostatic effects at alkaline pH higher than ~10.5: (i) increased electrostatic repulsion between neighbouring polypeptides of the complex, arising from increased net negative charge in the peripheral segments of these polypeptides, which can cause separation of the polypeptides from the complex; and (ii) ionization of residues such as tyrosine in the membrane-spanning α-helices, and neutralization of residues such as lysine which can bind to the negative membrane surface. These data imply that electrostatic stabilization may be significant in the specific assembly of the cyt b_6f complex in the thylakoid membrane.
Key words: alkaline pH extraction/chloroplast/extrinsic/intrinsic/membrane proteins

Introduction

The cytochrome (cyt) b_6f complex is an intrinsic, membrane bound protein complex that mediates electron flow between the photosystem II and photosystem I complexes in oxygenic photosynthesis (Cramer *et al.*, 1987).

It consists of four polypeptides of mol. wt > 15 000: cyt b_6, subunit IV (suIV) the Rieske iron-sulfur protein and cyt f. Cyt b_6 and suIV contain four (Szczepaniak and Cramer, 1990) and three (Widger and Cramer, 1991) membrane spanning α-helices, respectively; the Rieske FeS protein contains no (Hartl *et al.*, 1989) or 1-2 (Harnisch *et al.*, 1985; Steppuhn *et al.*, 1987; Schägger *et al.*, 1987; Willey and Gray, 1988; Usui *et al.*, 1990) transmembrane helices, and cyt f one such helix (Willey *et al.*, 1984; Szczepaniak and Cramer, 1989).

The folding and insertion of such integral protein complexes in the membrane bilayer has been proposed to be stabilized by (i) the hydrophobic and hydrophilic properties of the component amino acids and the main-chain hydrogen bonds that lead to the formation of independent stable trans-bilayer helices, and (ii) oligomeric complex formation through helix-helix, helix-lipid interactions within the bilayer, prosthetic group association and electrostatic interactions between interhelix loops that are exposed to the polar or aqueous phase (Singer, 1971; Engelman and Steitz, 1981; Engelman *et al.*, 1986; Popot and Engelman, 1990). The relative contribution of each of these components to the energy of stabilization in the membrane is not known. The role of the hydrophobic domain of membrane proteins in the energetic stabilization of the membrane structure has been emphasized (Kyte and Doolittle, 1982; Eisenberg, 1984; Engelman *et al.*, 1986; Yeates *et al.*, 1987).

The general consensus of the literature is that electrostatic interactions are relatively insignificant in the stabilization and organization of membrane proteins because of the lack of effect of ionic strength and alkaline pH on their apparent stability in the membrane (Singer, 1971). The ability of extreme alkaline pH or high ionic strength to remove 'extrinsic' proteins from membranes led to a clear experimental definition of extrinsic-peripheral and intrinsic membrane proteins (Singer, 1971; Steck and Yu, 1972). The present study shows that three of the four most hydrophobic large polypeptides of the b_6f complex, including the two with the largest number of membrane-spanning segments, are separately extruded from the thylakoid membrane at alkaline pH values in the range required to deprotonate lysine and tyrosine residues. This implies that electrostatic forces can be important in stabilizing oligomeric intrinsic membrane protein complexes.

Results

Sequential extrusion of the polypeptides of the cyt b_6f complex from the thylakoid membrane

The effect of extreme alkaline pH (> 10) on the association of polypeptides of the b_6f complex with the thylakoid membrane was tested initially in order to determine whether the Rieske iron-sulfur protein of the b_6f complex was an intrinsic membrane protein, or peripheral as proposed for the analogous protein in the mitochondrial bc_1 complex

(Hartl *et al.*, 1989). Incubation of membranes at extreme alkaline pH is a well documented procedure (Steck and Yu, 1972) for removing peripheral or extrinsic (Singer, 1971; Capaldi and Vanderkooi, 1972) proteins from membranes.

At pH values >11, the iron−sulfur protein was, indeed, released from the thylakoid membrane (Figure 1a, panel C; Figure 2) and appeared quantitatively in the supernatant (Figure 1b, panel C). However, the more hydrophobic cyt b_6 and subunit IV polypeptides were even more readily released from the thylakoid membrane, appearing in the respective supernatants after incubation at lower pH values (Figure 1a and b, panels A and B; Figure 2). The pattern

of appearance of these polypeptides in the respective supernatants complements that of their disappearance from the membrane, showing that the latter is not a consequence of degradation. The supernatants obtained after centrifugation at 30 000 g for 1 h were not turbid. The pH values at which 50% of the cyt b_6, subunit IV and iron−sulfur protein polypeptides were lost from the thylakoid membrane were ~10.7, 11.1 and 11.3, respectively (Figure 2). The loss of cyt f polypeptide from the membrane was small, even after incubation at pH 12. The calculated pI values of cyt b_6, suIV, the iron−sulfur protein and cyt f are 8.7, 7.3, 5.2 and 6.6, respectively (Widger and Cramer, 1991). The
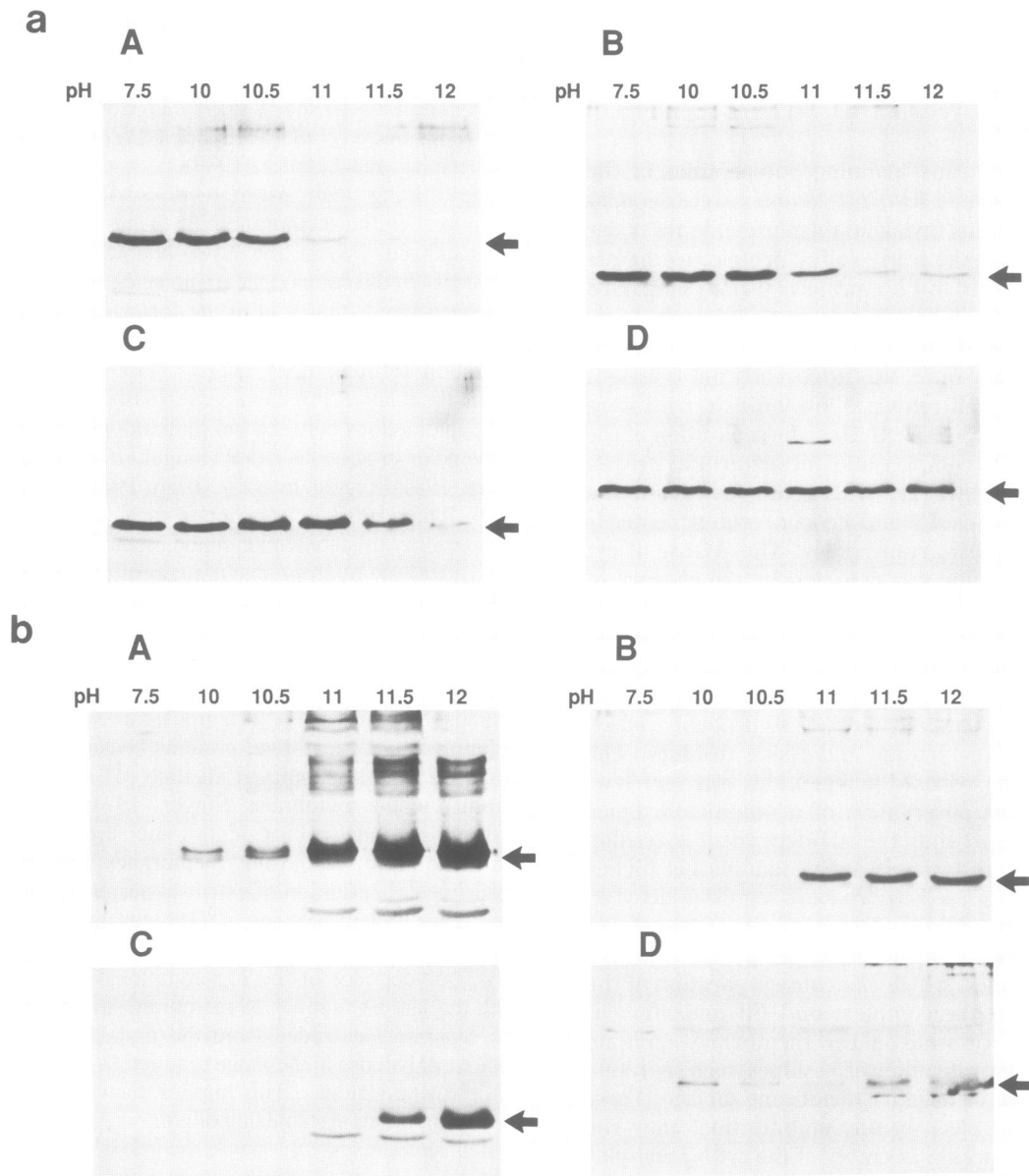
Fig. 1. Western blot analysis of the polypeptides of the b_6f complex extruded from the spinach thylakoid membrane at alkaline pH (10−12). (a) Polypeptides remaining in the membranes after incubation at different pH values. Thylakoids were washed with 10 mM HEPES, pH 7.5, containing 10 mM EDTA, and then incubated at pH 10, 10.5, 11, 11.5 or 12 for 30 min on ice. After centrifugation (30 000 g, 1 h), sediments were washed once with distilled water and analyzed for the presence of cyt b_6f polypeptides by immunodetection. Samples (equivalent to 10 μg Chl) were subjected to SDS−PAGE and analyzed by Western blotting using specific polyclonal antibodies against (A) cytochrome b_6 (arrow), (B) subunit IV (arrow), (C) Rieske iron−sulfur protein (arrow), and (D) cyt f (arrow). Control, thylakoids washed with 10 mM HEPES buffer, pH 7.5. (b) Western blot analysis of the supernatants resulting from the polypeptides of the b_6f complex extruded from the spinach thylakoids at alkaline pH (10−12). The supernatants were dialyzed against distilled water and lyophilized. Samples (equivalent to 10 μg Chl) were subjected to SDS−PAGE and analyzed by Western blotting with antibodies against cyt b_6 (A), subunit IV (B), Rieske iron−sulfur protein (C) and cyt f (D). Control, supernatant from thylakoids washed with 10 mM HEPES buffer, pH 7.5.

thylakoid membrane does not contain lipid head groups (e.g. phosphatidylethanolamine, phosphatidylserine, $R-NH_3^+$) with high pK (9−10) values.

The efficacy of alkaline pH in causing the extrusion of the cyt b_6 from the thylakoid membrane is further emphasized by comparison with the alkaline pH dependence of the removal of the extrinsic 16 kDa polypeptide from the oxygen-evolving complex (OEC) located on the lumen side of the thylakoid membrane (Figure 3A and B). A readily discernable fraction (~40−50%) of the 16 kDa polypeptide is membrane bound at pH 11 (Figure 3A and B), whereas ≤20% of the cyt b_6 remained bound at this pH (Figure 1a, panel A; Figure 2). Thus, cyt b_6 was somewhat more readily extruded from the thylakoid membrane than was the 16 kDa OEC peripheral protein. It is possible that the small difference in the pH dependence of the release is a result of the internal lumenal position of the 16 kDa polypeptide, as the large CF_1 ATPase subunits appear to be released at pH 10 (data not shown). The release of the internal 16 kDa polypeptide shows that the explanation for the retention of cyt f in the membrane fraction is not that it is released but that it is confined within the membrane lumen.

Cyt b_6 is released from the membrane in the apo form

Reduced minus oxidized difference spectra of the thylakoid membranes at pH 7.5 show the presence of the cyt b_6 hemes with a peak at 563−564 nm, and cytochromes b_{559} and f (peak at ~554 nm) in the dithionite−ascorbate (Figure 4A, spectrum 1) and ascorbate-ferricyanide (Figure 4A, spectrum 2) difference spectra. After incubation at pH 11, the main peak in the dithionite−ascorbate spectrum was found near 561 nm (Figure 4B, spectrum 1), indicative of the presence of low potential cyt b_{559} [reduced α-band maximum, 559.7 nm (Widger et al., 1984)], and some residual cytochrome b_6 in the membranes at pH 11, as indicated in the graph of Figure 2. The large change in midpoint potential of cyt b_{559} in damaged membranes from

a high potential state to lower potential states that are not reducible by ascorbate, but reducible by dithionite, is well documented (Cramer and Whitmarsh, 1977). The peak centered at 555 nm in the ascorbate-reduced spectrum (Figure 4B, spectrum 2) confirmed the presence of cyt f. These spectra are consistent with the extrusion of cyt b_6, but not most of the cyt b_{559}, from the membrane after incubation at alkaline pH values. Measurement of the dithionite-reduced difference spectra of the supernatant obtained at high pH from 200 μg/ml chlorophyll (Chl) (compared with the 80 μg/ml used for measurements of the membrane difference spectra) did not show the presence of any cyt b_6 heme (Figure 4B, spectrum 3), implying that cyt b_6 was in the apocytochrome form, and possibly unfolded when extruded from the membrane.

Reversible rebinding of the extruded polypeptides

The cyt b_6 and suIV polypeptides extruded into the supernatant at pH 11 could rebind to the thylakoid membranes if the pH of the suspension was subsequently shifted back to 7.5 or if the supernatant was separated from the thylakoids before the pH was shifted from 11 to 7.5, after which the two were recombined to assay binding (not shown). Because of the phenomena described above, it has not yet been possible to determine whether insertion into the membrane accompanied rebinding.

Extrusion of other thylakoid proteins at alkaline pH

SDS−PAGE gels of all thylakoid polypeptides present in the membrane after incubation at alkaline pH showed that other hydrophobic proteins besides cytochrome b_{559}, e.g. the prominent light-harvesting chlorophyll proteins (LHCPs), remain in the membrane at extreme alkaline pH values. Approximately 7% of the thylakoid membrane protein was
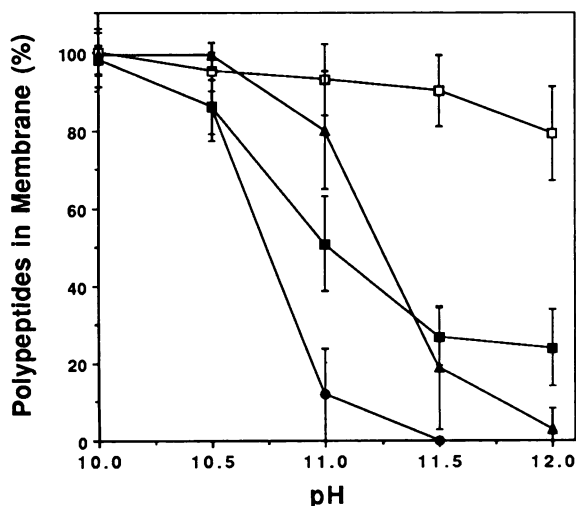
Fig. 2. Graph of the polypeptides from cyt b_6f complex remaining in the thylakoid membrane after alkaline pH (10−12) extrusion derived from the data shown in Figure 1. The relative protein concentrations were measured by densitometric analysis of photographic negatives of the Western blots. The points are expressed as percentages of the control sample and are the means of four different experiments. Epitopes of (●) cyt b_6, (■) subunit IV, (▲) Rieske iron−sulfur protein and (□) cyt f; standard deviations resulting from the average of four measurements are shown.
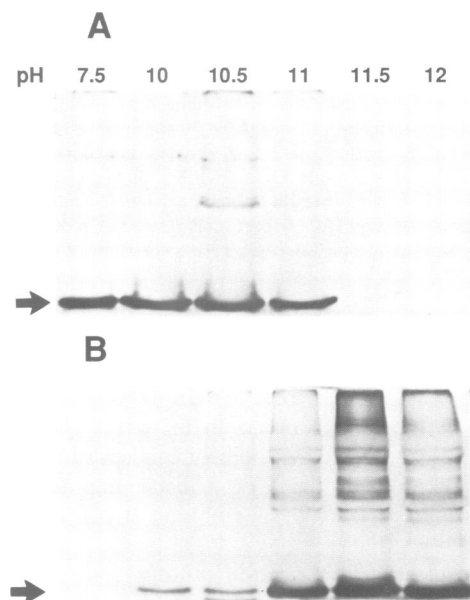
Fig. 3. Western blot analysis of the extrusion from the spinach thylakoid membrane at alkaline pH (10−12) of the 16 kDa extrinsic polypeptide (arrows) of the oxygen-evolving complex. Samples of the sediment (A) and the supernatant (B) were subjected to SDS−PAGE and analyzed by Western blotting with a polyclonal antibody against the 16 kDa polypeptide. Other conditions as in Figure 1a and b.

A

B
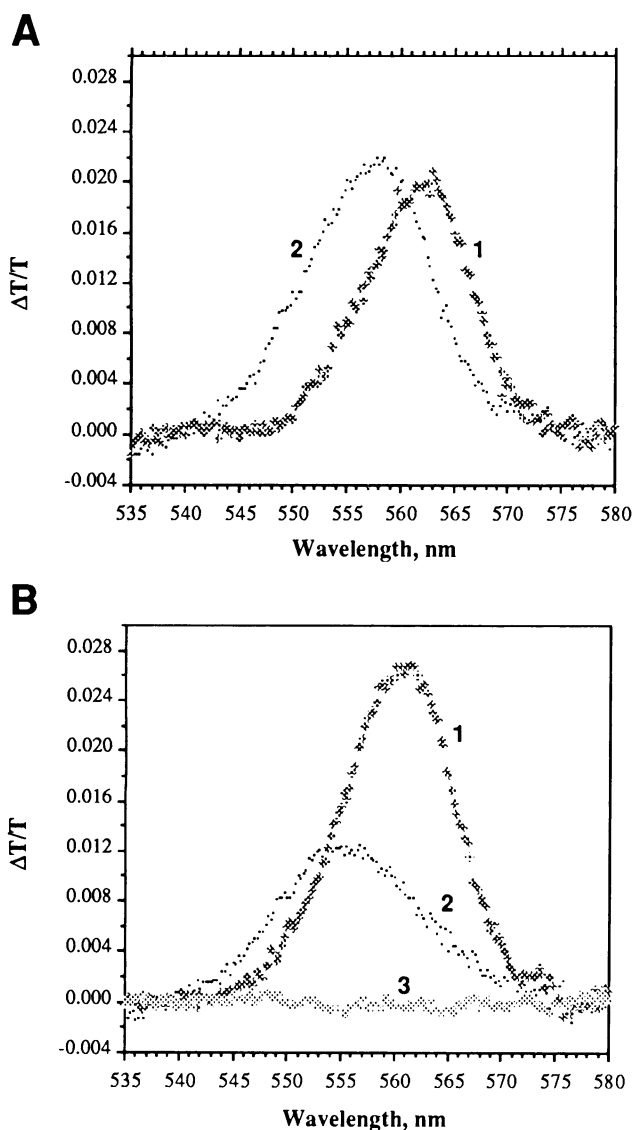
Fig. 4. Cytochrome α-band difference spectra of thylakoid membranes after incubation at different pH values. (A) Control: thylakoids washed with 10 mM HEPES buffer, pH 7.5; supernatant not shown.
(B) Thylakoids incubated at pH 11 as described in Materials and methods. Spectra of membranes in A and B: dithionite/anthraquinone-reduced minus ascorbate-reduced spectra (1); ascorbate-reduced minus ferricyanide-oxidized spectra (2); and, in (B), spectrum of the supernatant obtained after incubation at pH 11 (3).

found in the supernatant at pH 7.5, increasing to 17−28% as the pH was increased from 10 to 12 (Table I). At least 12 polypeptide bands were seen in SDS−PAGE of the supernatant arising from incubation of thylakoids at pH 11.5 (not shown). These include the prominent bands arising from the α and β subunits of the CF_1 ATPase that are mostly (>80%) removed from the membrane at pH 10.

Soluble nature of the extruded protein

To examine whether the extruded protein is in a membranous state, the protein contents of the sediment and supernatants of samples centrifuged at 100 000 g were measured as a function of pH. The sediment of the protein released at pH 10.5−12 contained a small amount of protein, <5% of the total protein released. Cyt b_6, suIV and the Rieske protein could either not be detected or only be barely detected in

Table I. Concentrations of protein and lipid released from the thylakoid membrane in the supernatant as a function of pH

Material in supernatant (μg/ml)	pH					
	7.5	10	10.5	11	11.5	12
Protein[a]	71	169	171	217	239	283
Lipid[b]	10.3	9.5	11.6	11.5	12.7	13.7
Free fatty acid[b]	3.4	2.7	3.1	5.3	8.0	9.5

[a]Protein determinations are the average of four assays done in duplicate; total thylakoid protein concentration, corresponding to 0.2 mg/ml Chl, was 0.98 mg/ml.
[b]Lipid and fatty acid measurements were done in triplicate and the range of numbers included in the averages did not exceed ±10%.

a Western blot of this sediment concentrated 20-fold (lane 2) relative to control membranes (lane 1) (Figure 5A−C). This demonstrated that the proteins of the b_6f complex released at alkaline pH were not in membrane vesicles, but rather in a micellar or soluble form.

Release of lipids and fatty acids at alkaline pH

Incubation of the thylakoid membranes at pH 77.5 (0°C, 8 min) resulted in the appearance of ~2.5% (10 μg /ml) of the thylakoid membrane lipid in the supernatant (Table I). The weight ratio of lipid to total protein in the supernatant was 1:7, 1:15 and 1:19 at pH 7.5, 10.5 and 11, respectively. This low lipid:protein ratio probably reflects the predominance in the supernatant of large peripheral proteins, such as the large CF_1 ATPase, which contain a small amount of bound lipid. Over the pH range 10.5−11.5 in which the three polypeptides of the b_6f complex were released from the membrane, the change in lipid concentration, approximately ±1 μg/ml, was at the detection limit of the measurements. The released lipid:protein ratio over this pH interval is ~1:30, so that the amount of lipid bound to the extruded b_6f complex [mol. wt = 60 000 for the three (b_6, suIV and iron−sulfur) polypeptides], is on the order of one lipid molecule per complex. Thus, the released polypeptides of the b_6f complex may contain a small amount of bound lipid, but they are not in a membranous nor apparently in a micellar state.

The pH dependence of release of fatty acids is of particular interest because of the possibility of lipid saponification and potential detergent action of the released fatty acids which might specifically extract the b_6f polypeptides from the membrane. The pH dependence of fatty acid release also shows a background level at pH 7.5 (3.4 μg/ml) (Table I). There is a small increase of ~2 μg/ml between pH 10.5 and 11.0, and a similar change between pH 11.0 and 11.5. It seemed unlikely that these small increases in fatty acid level might sequentially release cyt b_6 between pH 10 and 11, and then the suIV and iron−sulfur polypeptides between pH 11 and 11.5. This possibility was further examined by incubating thylakoid membranes at pH 7.5 with linolenic acid, the dominant fatty acid in thylakoid membranes, at a concentration of 20 μg/ml. The release pattern of cyt b_6 between pH 10.5 and 12 in the presence of linolenic acid was the same as that shown in Figure 2 and there was no release at pH 7.5 (data not shown).

Effect of high ionic strength

If the effect of high pH in causing release of the b_6f polypeptides from the membrane is to weaken an attractive
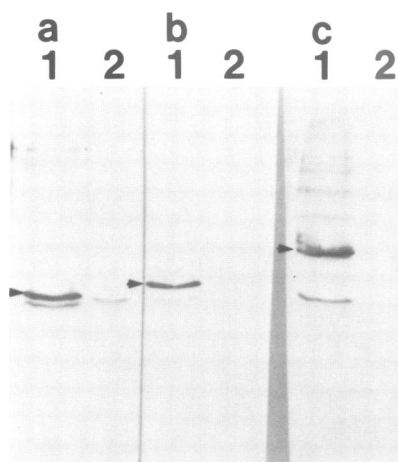
Fig. 5. Western blot analysis of the sediment resulting from centrifugation at 100 000 g (1 h) of the supernatants from alkaline extrusion at pH 11.5. Samples analyzed by Western blotting using antibody against (a) subunit IV, (b) Rieske iron–sulfur protein and (c) cyt b_6. Lane 1, control: sediment from thylakoids equivalent to 10 μg Chl; lane 2, sediment from supernatant obtained at pH 11.5, equivalent to 200 μg Chl.

Table II. Effect of NaCl concentration on extrusion from the thylakoid membrane of the polypeptides of the cyt b_6f complex at alkaline pH

NaCl concentration (M)	pH				
	10	10.5	11	11.5	12
	A. Cyt b_6 polypeptide remaining in membrane (%)[a]				
–[b]	99 ± 1	87 ± 8	6 ± 2	nd[c]	nd
0.15	100	94 ± 6	57 ± 27	38 ± 35	9 ± 10
0.6	100	95 ± 3	86 ± 3	60 ± 16	48 ± 24
	B. Subunit IV in membrane (%)				
–	99	86 ± 8	57 ± 15	27 ± 7	24 ± 10
0.15	95 ± 4	95 ± 2	78 ± 4	55 ± 18	30 ± 7
0.6	97 ± 4	90 ± 2	81 ± 3	77 ± 17	43 ± 19
	C. Rieske iron–sulfur protein in membrane (%)				
–	99 ± 1	99 ± 3	74 ± 13	15 ± 16	2 ± 5
0.15	100	95	72 ± 22	52 ± 46	9 ± 7
0.6	100	98 ± 3	84 ± 4	82 ± 10	48 ± 13

[a]Data for all three proteins in low ionic strength are the average of four to five experiments, and in 0.15 and 0.6 M NaCl the average of two experiments.
[b]Incubation medium in the absence of added NaCl consisted of an amount of NaOH sufficient to set the value of the pH.
[c]nd, not detectable.

electrostatic interaction with the membrane surface, then high ionic strength should cause polypeptide release, and should also cause the extrusion by alkaline pH to occur at lower pH values. However, incubation at higher ionic strength (e.g. 0.15 M or 0.6 M NaCl) did not cause release of the b_6f polypeptides at lower pH, but rather shifted the pH threshold for polypeptide release to higher values. The fraction of the cyt b_6 polypeptide remaining in the membrane at pH 11.0 was ~6% at low ionic strength and 86% in 0.6 NaCl, as determined by averaging the density of the Western blots (Table IIA). The fractions of suIV and the Rieske iron–sulfur protein remaining in the membrane at pH 11.5 under these ionic strength conditions were 27% and 77% (Table IIB) and 15% and 82% (Table IIC), respectively. At the higher ionic strength, the pH dependence of extrusion ot suIV and the iron–sulfur protein was similar (Table IIB and C).

Thus, increasing ionic strength did not facilitate the alkaline pH-induced extrusion of b_6f polypeptides, but rather acted to vitiate the effect of raising the pH. This suggests that the major effect of alkaline pH on polypeptide extrusion is to weaken repulsive electrostatic interactions of neighboring negatively charged peripheral polypeptide domains and possibly the interactions of these peripheral domains with the negatively charged membrane surface.

Discussion

Experimental definition of intrinsic versus extrinsic membrane proteins

Protein extraction from membranes at extreme alkaline pH or at low ionic strength is a consequence of increased coulombic repulsion. Incubation of membranes in 0.1 N NaOH is a well-known procedure for removal of peripheral proteins (Steck and Yu, 1972). It has been proposed that electrostatic interactions are relatively insignificant in the stabilization and organization of intrinsic membrane proteins (Singer, 1971). It was therefore unexpected that the three

intrinsic hydrophobic polypeptides of the cytochrome b_6f complex could be sequentially extruded from the membrane in the pH range 10–12, with the two most hydrophobic polypeptides extruded most readily.

Two stages in the extrusion process

An intact and active cytochrome b_6f complex containing four polypeptides with $M_r > 15\ 000$ can be purified in detergent as a hydrophobic intrinsic membrane protein complex (Hurt and Hauska, 1981; Black *et al.*, 1987). The sequential release of these polypeptides from the thylakoid membrane includes at least two stages: (i) separation of the polypeptides of the complex in the membrane, and (ii) sequential extrusion from the membrane. The fact that the extrinsic, lumen-side OEC 16 kDa polypeptide and the iron–sulfur protein are released from the membrane implies that the reason for non-release of cyt f is not that it is entrapped on the lumen side, but rather that cyt f is not released from the membrane. The separation of the polypeptides of the complex can be ascribed to electrostatic repulsion.

Origin of the electrostatic repulsion

The increase in net negative charge in the peripheral segments of the cyt b_6f polypeptides that occurs upon increasing the pH from 7.5 to 11 can be estimated from topographical models (cf. Figure 6A for cyt b_6) (Szczepaniak and Cramer, 1990; Widger and Cramer, 1991), and assumed pK values in proteins for Arg, Lys and Tyr residues of 12–12.5, 10–10.8 and 9.7–10.1, respectively (Schulz and Schirmer, 1979; Creighton, 1983). Charge changes of Arg residues, whose pK values are too high to be affected by pH changes in the range 10–11.5, were not included in the calculation. The peripheral segments of cyt b_6 on the stromal and lumenal sides of the membrane would carry a net charge of approximately +1 and 0 at pH 7.5, and −6 and −2.5 at pH 11, assuming that the charge on
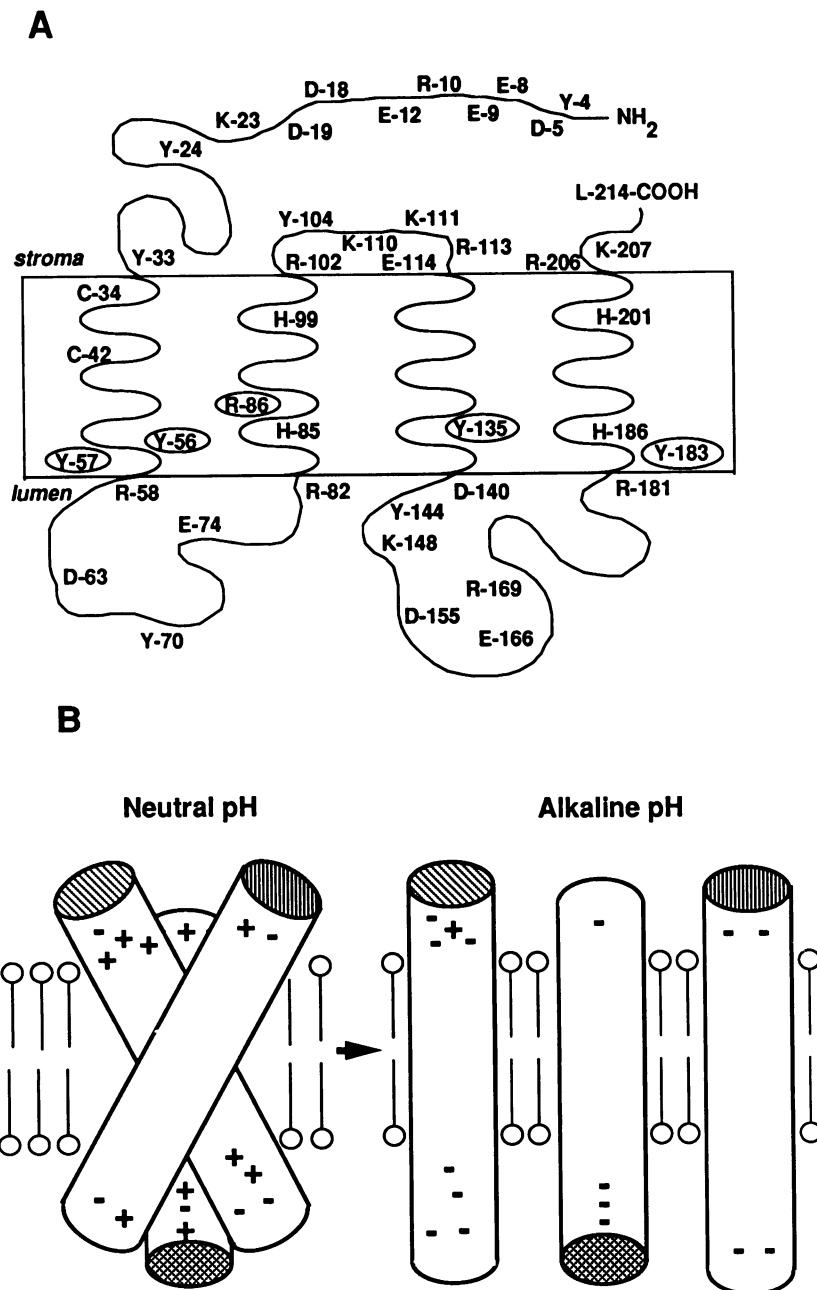
Fig. 6. (A) Folding of the cytochrome b_6 polypeptide in the thylakoid membrane bilayer (Szczepaniak and Cramer, 1990), showing residues that are probably charged at pH 7.5 or 11. Potentially ionizable residues in the membrane-spanning α-helices are circled. (B) Diagram of separation of polypeptides of an oligomeric intrinsic membrane protein complex due to electrostatic repulsion at alkaline pH of negative charges in the peripheral domains.

a Lys and Tyr residue at pH 11 is 0.5 and -1 respectively. The net change in charge of cyt b_6 in the peripheral region on both sides of the membrane in the pH 7.5 to 11 transition would be aproximately -10 charge units. If one assumes three helix and one helix models for suIV and the iron—sulfur protein, respectively, with the NH$_2$-terminus for each protein on the stromal side of the membrane (Widger and Cramer, 1991), the net charge on the stroma and lumen sides of suIV would decrease from $+4$ and -4 at pH 7.5 to -1.5 and -6 at pH 11, a change of approximately -8 electronic charges. For the iron—sulfur protein, the charge on the stroma and lumen sides would decrease from 0 and -2 at pH 7.5, to -1.5 and -7 at pH 11, a net change of approximately -7 charge units. The membrane-spanning helix of the iron—sulfur protein was assumed to start near

Thr17 (Widger and Cramer, 1991) in order to account for its insensitivity to trypsin added from the stromal side (A.Szczepaniak and W.A.Cramer, unpublished). The above calculation of negatively directed charge changes is approximate and is intended to illustrate the large negatively directed charge changes in the peripheral loop regions of such integral membrane polypeptides which could cause their separation in the membrane (Figure 6B).

At pH 7.5, (i) most of these peripheral charges are electrostatically compensated, and (ii) at physiological pH, cyt b_6 and suIV contain a smaller number of positive charges on the lumen (translocated) side of the membrane. This topography agrees with the '*cis*-positive' rule proposed by von Heijne (1988) for intrinsic membrane proteins with peripheral loops containing ≤ 70 residues.

The role of electrostatic forces stabilizing oligomeric membrane protein complexes

It has been proposed that interaction of the membrane-spanning helical domains may be sufficient to account for self-assembly of intrinsic membrane proteins and protein complexes that span the bilayer several times (Popot and Engelman, 1990). The present studies imply that the association of the polypeptides of the cytochrome b_6f complex is also mediated by electrostatic interactions. These interactions become repulsive at the alkaline pH region above typical tyrosine and lysine pK values. The strength of the repulsive interactions is apparently large enough to cause dissociation of the b_6f polypeptides in the thylakoid membrane.

The release of the b_6f polypeptides at alkaline pH is probably not aided by a favorable free energy change (ΔG) for the folded → unfolded transition in aqueous solution. if a marginal stability is assumed for the folded form of globular proteins in solution at neutral pH (Kim and Baldwin, 1982; Engelman et al., 1986), then the transition of the helix in the bilayer to the random coil in solution would be associated with a positive ΔG.

Forces responsible for extrusion from the membrane

It is proposed that extrusion of the cyt b_6f polypeptides results (i) from residues in the membrane bilayer that are ionizable at alkaline pH, and (ii) possibly from the loss of net positive charge that could be involved in binding to the negative surface of the membrane [the charge density on lumen and stromal surfaces is approximately -0.034 and -0.025 C/m² in 5 mM KCl (Barber, 1982)].

(i) Using the existing topographical models for the b_6f polypeptides in the membranes of chloroplasts and cyanobacteria (Widger and Cramer,. 1991), the potentially ionizable groups in cyt b_6 (circled in Figure 6A) are: two to three Tyr and possibly two Cys residues in helix A, Tyr135 in helix C which could hydrogen bond to the unique Arg86 in helix B, and Tyr183 in helix D. SuIV contains Tyr252 and Cys264 in helix A (of three helices), and the iron−sulfur polypeptide contains Tyr32 and Tyr37 in the proposed single membrane-spanning α-helix (Widger and Cramer, 1991). Cyt f, which is not extruded, has no potentially ionizable residue in the COOH-terminal membrane-spanning segment Val251 → Leu270 in the spinach chloroplast sequence (recent sequence summary in Widger and Cramer, 1991). The cyt b_{559} α-subunit, which is not extruded, has a Tyr residue at each end of a 26 residue non-polar segment that is longer than necessary to span the membrane (Tae et al., 1988), and it is assumed that the side chain of each of these terminal Tyr residues is in the aqueous phase. The LHC IIb polypeptide (helices A−C), part of the non-extruded LHC family, appears to have only one potentially ionizable Cys residue that resides on the helix nearest the NH₂-terminus. We would propose that this Cys residue is not ionizable at high pH or that the LHC is stabilized in the membrane bilayer by the many (15) bound chlorophyll molecules (Kühlbrandt and Wang, 1991).

(ii) The zeta potential in 10 mM NaCl for membrane vesicles containing the 20−25 mol % negative lipid characteristic of thylakoid membranes (Gounaris et al., 1983), is approximately −90 mV (McLaughlin, 1989). This implies that the stabilization energy of an individual charge of a basic Lys or Arg residue positioned at this potential would be about −2.0 kcal/mol. The loss in stabilization

energy would be ~3 kcal/mol at pH 11 for the loss of 0.5 positive charge on a lysine and an increase of one negative charge on a similarly positioned tyrosine.

Nature of the membrane-bound state of the Rieske iron − sulfur protein

The initial purpose of testing the effect of alkaline pH on the thylakoid membrane was to determine whether the Rieske iron−sulfur protein was intrinsically (Steppuhn et al., 1987; Willey and Gray, 1988) or peripherally (Hartl et al., 1989) bound. The iron−sulfur protein could be released from the membrane at alkaline pH. However, the required pH was somewhat extreme (pH for half-release > 11) and the more hydrophobic cyt b_6 and suIV polypeptides were released at lower pH values. Thus, it was concluded that the Rieske iron−sulfur protein is an intrinsic membrane protein, although it is not possible from the present data to discriminate definitely between one and two membrane-spanning α-helices.

Materials and methods

Preparation of thylakoids and polypeptide extrusion from thylakoids at alkaline pH

Spinach leaves (50 g) were taken from plants grown on a 12 h light 12 h dark cycle, homogenized in 250 ml buffer (0.3 M sucrose, 10 mM NaCl, 50 mM HEPES, pH 7.5) for ~5 s, filtered through four layers of cheesecloth, then centrifuged (1000 g, 3 min, stopped by hand). The sediment was resuspended in the same buffer, centrifuged (1000 g, 30 s), the sediment discarded and the supernatant centrifuged (3000 g, 5 min). The resultant centrifuge pellet was resuspended in 5 mM HEPES−NaOH (pH 7.5), 10 mM EDTA at a chlorophyll concentration of 0.4 mg/ml, and centrifuged (17 000 g, 15 min). The discarded supernatant was free of chlorophyll. The centrifuge sediments were resuspended at a concentration of 0.2 mg/ml in distilled water adjusted with NaOH, or 5 mM CAPS [3-(cyclohexylamino)-1-propanesulfonic acid]−NaOH, at pH 10, 10.5, 11, 11.5 and 12. The pH was checked after addition of chloroplasts and also in supernatants obtained after incubation for 5−30 min and subsequent centrifugation. As a control, chloroplasts were incubated in 10 mM HEPES−NaOH, pH 7.5, for 30 min at 0°C, after which the thylakoids were separated by centrifugation (30 000 g, 0.5−1 h). After centrifugation, the supernatants were clear and not opalescent below pH 11, and very slightly yellow-green and non-turbid between pH 11 and 12. After centrifugation at 100 000 g (1 h), the supernatants were non-turbid and colorless. The sediments of the 30 000 g centrifugation were washed once with distilled H₂O, subjected to SDS−PAGE and analyzed by Western blotting. Similarly, the supernatants from alkaline extrusion were dialyzed against distilled H₂O, lyophilized and subjected to SDS−PAGE, and analyzed by Western blotting. Protein concentrations were determined by the Micro-BCA (bicinchoninic acid, Pierce Chemical Co.) method.

SDS − PAGE and Western blotting

These procedures are as described by Szczepaniak and Cramer (1990), except that Western transfer was carried out using a current of 135 mA for 30 min using a semi-dry transfer unit (Hoefer).

Chemical difference spectra of cytochromes

Spectra were obtained as described by Furbacher et al. (1989). Chloroplasts were washed in distilled H₂O after incubation at varying pH values, and suspended for all spectrophotometric measurements at a concentration of 80 μg/ml Chl in 0.2 M sucrose, 30 mM Tricine−KOH, pH 7.8, 5 mM MgCl₂ and 25 mM KCl.

Lipid and fatty acid determination

Lipids were extracted according to Kates (1972). TLC lipid separations were on Whatman HP-K, 10×20 cm plates, which were prewashed by development in chloroform−methanol (2:1, v/v) and dried under vacuum. Non-polar ('neutral') lipids were separated in hexane: diethyl ether:acetic acid (44:6:0.5 by volume). Polar lipids were separated in methyl acetate:n-propanol:chloroform:methanol:0.25% aqueous KCl (25:25:28:10:7 by volume) (Heape et al., 1985). Separated constituents were rendered visible by dipping plates into 3% cupric acetate (w/v) in 8% (v/v) aqueous phosphoric acid and heating for 15 min at 180°C (Fewster et al., 1969).

TLC plates for fatty acid determination were developed in hexane:diethyl ether:acetic acid (88:12:1). Plates were charred with cupric acetate—phosphoric acid as above and the band corresponding to fatty acids was scanned with a densitometer. For a standard, known amounts of linoleic acid (18:2) were separated and scanned. Total fatty acid in thylakoid membranes was determined after saponification in a 1:1 mixture of chloroform—0.6 N methanolic KOH for 90 min at 65°C in a sealed tube. After acidification, non-saponifiable lipids were recovered, streaked onto plates which were developed, charred and scanned as above. The regions corresponding to fatty acids and methyl esters were scanned.

Acknowledgements

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