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Transverse organization of components within the chloroplast cytochrome *b*-563 / *f* complex

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The transverse organization of four major components of the chloroplast cytochrome *b*-563 / *f* complex from pea and spinach leaves was investigated. The effects of proteolytic digestion of well-defined inside-out and right-side-out vesicles was determined by two independent methods. These were (i) investigation of haem-associated peroxidase activity of samples subjected to mildly denaturing SDS-polyacrylamide gel electrophoresis, and (ii) analysis of the interaction of antibodies raised against components of the cytochrome complex with samples subjected to SDS-polyacrylamide gel electrophoresis. A complementary approach involved adsorption of antisera with untreated vesicles and subsequent analysis of the antisera with Western blotting techniques. It was shown that cytochromes *f* and *b*-563, the Rieske iron-sulphur protein and an M_r 17000 component all span the membrane and that all have their carboxyl-terminal end protruding into the stromal matrix. However, the two cytochromes and the Rieske iron-sulphur centre were most readily accessible to added agents at the lumenal surface. These results are discussed in the light of recently published amino acid sequence data for cytochromes *f* and *b*-563.

Introduction

The chloroplast cytochrome *b*-563/*f* complex is primarily responsible for electron transfer between Photosystems I and II [1–3]. The transmembrane nature of this electron flow is an essential feature of the energy-transducing reactions of photosynthesis. In order to have a full understanding of these processes, it is essential that we have a clear model of the cytochrome complex arrangement within thylakoids. In its minimal functional form, the complex appears to consist of 1 cytochrome *f*, 1 cytochrome *b*-563 (with two haem groups), 1 Rieske iron-sulphur protein, one each of an M_r 17000 and an M_r 5000 polypeptide and one apparently bound quinone molecule [4]. It is the vectorial arrangement of the first four of these components to which we address ourselves here.

Sealed, sub-mitochondrial vesicles with exposed inner or outer membrane surfaces have been available for many years [5]. In combination with specific proteinases and antibodies, these vesicles have been successfully used to study membrane topography. Formerly, the use of chloroplast membranes disrupted by sonication or by detergents was unsatisfactory, since the resulting exposed surfaces were uncharacterized and gave ambiguous results. The current availability of sealed, inverted thylakoid vesicles [6] has greatly improved this position. Recently, Willey et al. [7] and

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Abbreviations: Chl, chlorophyll; Cyt, cytochrome; PS, Photosystem; SDS, sodium dodecyl sulphate.

Mansfield and Bendall [8] have independently shown that cytochrome *f* is, in part, exposed at the intrathylakoid lumenal surface. Analysis of amino acid sequence data led the former group [7] to suggest that the major part of cytochrome *f*, including the haem group, is located within the lumen. Such a large protruding unit has been detected by electron microscopic analysis of reconstituted liposomes [9]. However, Mansfield and Bendall [8] suggested that only a small fraction of the total protein is exposed and that the haem group is located in a relatively protected environment. A model of cytochrome *b*-563 arrangement within the membrane has been put forward on the basis of a hydropathy index plot of its amino acid sequence [10]. It was suggested that cytochrome *b*-563 is largely buried within the membrane, as has been indicated by numerous biochemical studies (reviewed in Ref. 11). However, the model [10] also predicts that many sections of the protein, including the amino and carboxyl termini, extend into the stromal or lumenal regions. This has yet to be experimentally demonstrated. There is little evidence concerning the function or organization of the M_r 17000 protein, except that it may be homologous to the carboxyl-terminal end of the analogous *b*-type cytochrome found in mitochondria [10].

Numerous studies of the Rieske iron-sulphur centre in mitochondrial membranes have indicated that the protein is located near the outer membrane surface adjacent to cytochrome c_2 [1]. A similar location was proposed for the Rieske protein in *Rhodopseudomonas sphaeroides* following experiments in which paramagnetic ions were used to perturb the inherent electron paramagnetic resonance signal of the iron-sulphur centre in differently oriented membranes [11]. By deduction, it seems likely that the inner thylakoid surface (which is analogous to the outer surface of the inner mitochondrial membrane, see Ref. 1) is the site of the chloroplast Rieske centre in higher plants. In support of this hypothesis, it has been shown that a specific antibody to the protein inhibited the catalytic activity of the isolated cytochrome complex but did not inhibit electron transport in unbroken thylakoids [12]. This result, however, does not allow us to distinguish clearly between protein exposure at the two thylakoid surfaces or a location within the membrane.

In this study, we used thylakoid membrane vesicles of well-defined and opposite sidedness to investigate the topography of the four main components of the chloroplast cytochrome complex. It was shown that all four components (the two cytochromes, the Rieske iron-sulphur protein and the M_r 17000 protein) span the membrane. Cytochrome *f* and the Rieske protein were found to be located mainly at the inner surface. The carboxyl termini of all four components (previously demonstrated only for cytochrome *f* [7]) were shown to be exposed to the stromal matrix.

Materials and Methods

Membrane preparation. Inside-out and right-side-out vesicles were prepared from the leaves of pea seedlings (*Pisum sativum*, Greenfeast variety) and hydroponically grown spinach (*Spinacea oleracea*) by the aqueous polymer two-phase partition method described in Ref. 6 and modified as in Ref. 13 using dextran and poly(ethylene glycol) concentrations of 5.7% (w/w). The orientation of the membrane preparations was ascertained by measurements of proton extrusion (inside-out vesicles) or uptake (right-side-out vesicles as described in Ref. 13.

Proteinase treatment. Partial proteolysis was carried out as described previously [8] using trypsin (bovine pancreas, type III; 10000 BAEE units/mg solid at 25°C) or carboxypeptidase A-PMSF (bovine pancreas, type 1-PMSF; 67 units/mg protein where one unit will hydrolyze 1.0 μmol hippuryl-L-phenylalanine per minute at pH 7.5 and 25°C). Other samples were treated with leucine aminopeptidase (porcine kidney, type III-CP cytosolic; 200 units/mg protein where one unit will hydrolyze 1.0 μmol of L-leucinamide to L-leucine and NH_3 per minute at pH 8.3 and 25°C) in a medium containing 5 mM $MgCl_2$ and 0.1 M Tris (pH 8.0) at 25°C. All proteinases were obtained from Sigma Chemical Company. Tryptic digestion was stopped and membrane samples were recovered as described in Ref. 8. Carboxypeptidase activity was inhibited by addition of 4 mM *o*-phenanthroline to the diluting medium; similarly aminopeptidase activity was inhibited by 5 mM ethylenediaminetetraacetic acid (disodium salt) prior to membrane recovery. Control samples were

treated in the same way but without the initial addition of proteinase.

Preparation and use of antigens and antibodies. Cytochrome *b*-563/*f* complex was prepared according to the method of Hurt and Hauska [14]. Spectroscopic and electrophoretic analysis indicated a minimal chlorophyll content, absence of cytochrome *b*-559 and a polypeptide profile similar to that given in Ref. 14 (results not shown). Antibodies were raised against the complex in rabbits by subcutaneous injections of samples containing 200 μg protein in distilled water homogenized with an equal volume of Freunds adjuvant (1 ml total). Samples of cytochrome complex (4 mg protein total) were separated on 3-mm-thick Laemmli-type gels [15]. Gels were then stained with Coomassie blue, destained and bands corresponding to cytochrome *f*, cytochrome *b*-563, the Rieske protein and the M_r 17000 protein were excised. These were finely cut, frozen in liquid nitrogen, ground to a powder and resuspended in a medium containing 0.9% NaCl, 10 mM Tris-HCl (pH 7.4) before injection into individual rabbits. Injections were repeated at monthly intervals and the rabbits were bled from the marginal ear vein 5 weeks after the initial injection, and monthly thereafter.

For membrane/antibody interaction experiments samples were resuspended in a solution containing 0.9% NaCl, 10 mM Tris-HCl (pH 7.4) and 3% bovine serum albumin. Aliquots of antisera were then added to give mg Chl : μl antiserum ratios as detailed in the figure legends. Membranes were removed from suspension by centrifugation (35000 × *g* for 90 min). Electrophoretic blotting of untreated or proteolytically degraded samples from SDS-polyacrylamide gels onto nitrocellulose sheets was carried out as in Refs. 16 and 17. Subsequently, the blotted material underwent sequential reaction with complete or extracted antisera and ^{125}I-labelled protein A (from Amersham, 30 mCi/mg) prior to autoradiography [17,18].

Polyacrylamide gel electrophoresis. In order to prepare samples for polypeptide analysis or for electroblotting onto nitrocellulose, the method of Laemmli [15] was used, as described in ref. 8, except that samples were boiled for 1 min in solubilizing buffer prior to electrophoresis. This was necessary because residual chlorophyll bound to protein prevented uniform access of antibody to the blot and gave anomalous banding patterns on autoradiographs. In order to detect haem-associated peroxidase activity from cytochrome *b*-563, samples were prepared and eletrophoretically separated on mildly dissociating SDS slab gels for 3 h at 30 mA and 0°C according to the procedures of Machold (see Ref. 19), except that an 8–12% acrylamide gradient was used. Peroxidase activity in gels was then detected as described in Refs. 20 and 21. Staining intensity was determined using a Varian-Techtron 635 scanning densitometer set at 680 nm. Each gel slice was scanned in two directions and the integrated area under the peaks was used as an indication of concentration.

Results

The vesicle preparations were highly reproducible, as determined by two main parameters. The average Chl *a*/*b* ratios (lowering of which represents PS II enrichment) of samples from pea leaves were as follows: chloroplasts, 2.49 ± 0.13; inside-out vesicles,1.95 ± 0.11; right-side-out vesicles, 2.55 ± 0.15 (± standard deviation of 34 individual preparations). The corresponding values from spinach leaves were: 2.58 ± 0.09, 1.96 ± 0.12 and 2.75 ± 0.20, respectively (15 individual preparations). The proton-pumping activity of the pea (and spinach) fractions was determined by the extent of the pH change in suspensions upon illumination. The equilibrium values were as follows: inside-out vesicles, 40 ± 3 nmol/mg Chl (44 ± 3 nmol/mg Chl)—extrusion; right-side-out vesicles, 45 ± 4 nmol/mg Chl (47 ± 3 nmol/mg Chl)—uptake: each value is the average of five measurements (three in the case of spinach fractions).

Proteolysis studies on haem-containing proteins

(a) Effects of trypsin. The effects of proteinases on haem-containing membrane proteins, such as cytochromes, can be followed by staining SDS-polyacrylamide gels for haem-associated peroxidase activity [20,21]. This has been successfully carried out for cytochrome *f* [8]. However, the non-covalently bound haem groups of cytochrome *b*-563 were displaced during electrophoresis and were not detected. By using a mildly dissociating

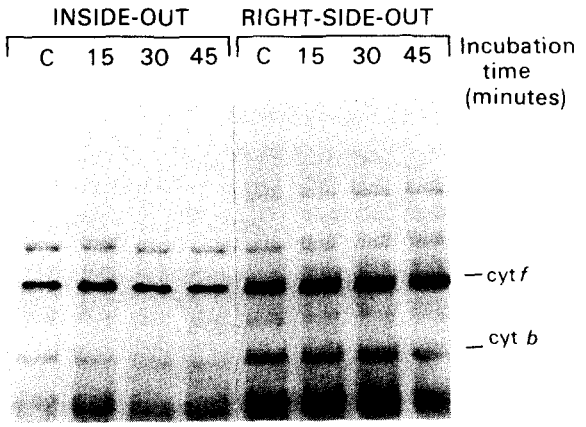

Fig. 1. The effect of trypsin digestion on the haem-associated peroxidase activity of cytochromes *f* and *b*-563 in right-side-out and inside-out vesicles prepared from pea thylakoids. Samples were treated as a Chl : trypsin ratio of 10 : 1 (mg : mg) at 20°C for the times indicated; control samples (C) were maintained at 20°C for 45 min in the absence of proteinase.

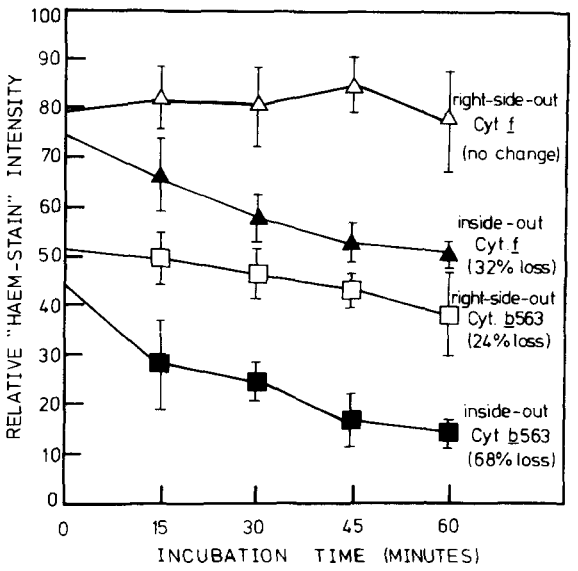

Fig. 2. Densitometric analysis of gels similar to that in Fig. 1. Error bars indicate the standard deviations of readings from five separate experiments. Gels were scanned at 680 nm.

gel system, the effect of trypsin on the 'haem-staining' pattern of cytochrome *b*-563 could be followed (Fig. 1). The unlabelled bands were green, chlorophyll-retaining proteins as detailed in Ref. 19. There was never any 'free haem' observable at the electrophoretic front, although this does not indicate that there was no haem loss from the cytochromes. However, the intensity of 'haem-staining' was reproducible from gel to gel, as shown in Fig. 2, which gives the average stain intensity due to the two cytochromes in five similar experiments. From the gel it can be seen that the intensity of staining associated with cytochrome *b*-563 in inside-out vesicles progressively decreased. A 68% loss occurred within the measured time, compared with a 24% loss from right-side-out vesicles. It was shown in Ref. 8 that pronase causes a more extensive loss of cytochrome *b*-563, as measured spectrophotometrically, from the inner rather than the outer surface of the membrane. Routinely, a slight loss of mass was observed from this cytochrome, as may be observed if the 45 min treated inside-out sample (Fig. 1) is compared with the control right-side-out sample. However, the gel was run over only a short distance in order to minimize haem loss, and the change in mobility could not be strictly quantified. Such a change in mobility would be consistent with the location of trypsin-susceptible arginine and/or lysine residues near the amino or carboxyl termini of the protein [10]. The model of cytochrome *b* proposed by Widger et al. [10] indicates that such residues may exist at either side of the membrane on hydrophilic sections of the protein. Cleavage of such bonds on sections linking the haem-binding groups of membrane spanning regions II and V would probably lead to loss of haem, and hence decreased stain intensity, on the subsequent gel.

As noted previously in Ref. 8, there was no detectable change in the mobility of cytochrome *f* upon exposure of either membrane surface to trypsin for pea or spinach thylakoid vesicles. A 32% loss of stain intensity from cytochrome *f* was detected in inside-out vesicles only (Fig. 2).

(b) Effects of carboxypeptidase. This enzyme progressively cleaves the carboxyl termini from susceptible proteins. Right-side-out and inside-out thylakoid vesicles prepared from both pea and spinach leaves were incubated with carboxypeptidase, and subsequently analysed for haem content on mildly dissociating gels as described earlier. The proteinase had an effect on both cytochromes in right-side-out vesicles only (Fig. 3). We were able to confirm the report in Ref. 7 that cyto-

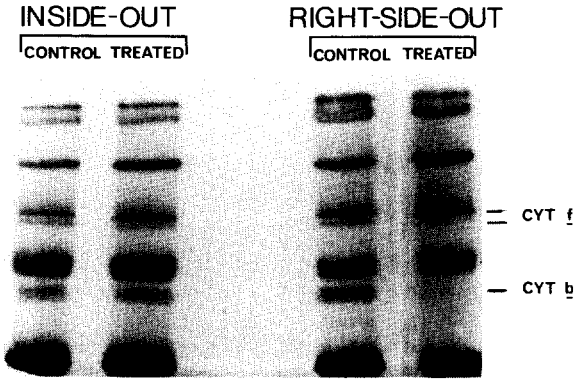

Fig. 3. The effect of carboxypeptidase on the haem-associated peroxidase activity of cytochromes *f* and *b*-563 in right-side-out and inside-out vesicles prepared from pea thylakoids. Samples were treated at a Chl:proteinase ratio of 10:1 (mg:mg) at 20°C for 30 min. Control samples were treated in the same way but in the absence of proteinase. Samples were then subjected to electrophoretic separation under mildly dissociating conditions for 3 h at 50 mA and 0°C.

chrome *f* loses an M_r 1000 fraction upon such treatment. This was more evident in gels that were run over longer distances. The samples shown here were electrophoresed rapidly (3 h) over a short distance (6 cm) in order to minimize loss of haem groups from cytochrome *b*-563. The stain associated with the haem of cytochrome *b*-563 is almost completely lost from carboxypeptidase-treated right-side-out vesicles. The same band in inside-out vesicles lost 33% stain intensity compared with the untreated control. This loss may be attributed to the approx. 30% contamination of the inside-out fraction by right-side-out vesicles [6]. Widger et al. [10] proposed that the carboxyl terminus of cytochrome *b*-563 is a continuation of the membrane-spanning section V, upon which both haem groups have a coordinating histidine residue. Disruption of this section of the protein by the carboxypeptidase may well have led to loss of the non-covalently bound haem groups, as observed, rather than a change in mobility of the haem-containing band. We were unable to observe any significant change in position of the protein itself (by subsequent staining with Coomassie blue) because of the complexity of the polypeptide pattern in this region of the gels.

Antibody studies

(a) Effects of trypsin. Trypsin-treated inside-out or right-side-out membranes were subjected to denaturing SDS-polyacrylamide gel electrophoresis and the separated polypeptides were then electroblotted onto nitrocellulose paper. It was then possible to probe samples with an antiserum raised against the isolated cytochrome complex. The upper band on the resulting autoradiograph (Fig. 4) corresponds to cytochrome *f* and shows that the protein is largely resistant to trypsin at either surface. The faint, central bands are probably the result of antibodies raised against residual traces of light-harvesting complex contaminating the cytochrome complex, since the well-known change in mobility of this protein upon trypsin treatment [22] can be detected. The lowest band in Fig. 4 corresponds to the Rieske iron-sulphur protein and not to cytochrome *b*-563, as determined by staining an adjacent track of the original gel with Coomassie blue. Similarly, when a mildly denaturing gel system was used, a subsequently 'haem-stained' cytochrome *b*-563 band did not align with

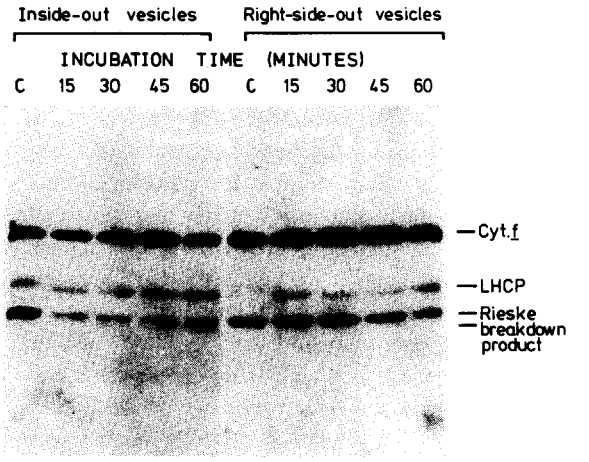

Fig. 4. The effect of trypsin digestion on cytochrome *f* and the Rieske iron-sulphur protein from pea thylakoid vesicles. Samples were treated at a Chl:trypsin ratio of 10:1 (mg:mg) at 20°C for the times indicated; control samples (C) were maintained at 20°C for 60 min in the absence of proteinase. Membranes were electrophoretically separated under denaturing conditions and blotted onto nitrocellulose paper before incubation with anti-cytochrome *b*-563/*f* complex antiserum and ^{125}I-labelled protein A. The autoradiograph was exposed for 48 h at 193 K. (LHCP, antigenic reaction against light-harvesting chlorophyll protein.)

any of the bands on an autoradiograph equivalent to that in Fig. 4 (results not shown). It is clear from Fig. 4 that the Rieske protein rapidly lost a significant proportion (70–80%) of its original antigenicity upon trypsin treatment of inside-out thylakoids. Following the initial change there was no further decrease in the extent of antibody interaction with the blotted protein. However, a faint band did appear about M_r 1000 below the Rieske protein. The latter protein was not visibly affected in the right-side-out fraction until the 60 min sampling, when a slight decrease in interaction with the antibody was observed.

We suggest that these results indicate the presence of a highly antigenic, trypsin-labile fraction of the Rieske protein at the inner surface. The residual antigenic material at the M_r 20000 position (Fig. 4, inside-out membranes treated for 15–60 min) is due to unaffected protein that was present in contaminating right-side-out vesicles. The origin of the band that appeared at M_r 19000 cannot be conclusively determined from these data but may represent breakdown products from cytochrome *f*, since the appearance of the band was progressive and did not correspond with the rapid disappearance of the Rieske protein.

(b) Effects of carboxypeptidase. Inside-out and right-side-out vesicles derived from both pea and spinach leaves were treated with carboxypeptidase and analysed using antibodies raised against the cytochrome complex, as detailed in the preceding section. The antibodies used in these experiments were raised against spinach cytochrome complex but they react equally well with the corresponding proteins from pea (Fig. 5). In each case, the carboxypeptidase digested an M_r 1000 fraction from cytochrome *f* in right-side-out vesicles only, thus confirming the orientation proposed by Willey et al. [7]. The Rieske protein of both species was also affected by the carboxypeptidase at the outer membrane surface only. However, this was manifested by a decrease in antibody interaction with the nitrocellulose-blotted sample rather than a change in mobility. This suggests that the fraction removed from the protein may have consisted of only a few amino acids, but that these were highly antigenic. In Fig. 6, we show the result of incubation of an antiserum raised against the M_r 17000 polypeptide (excised from gel slices) with

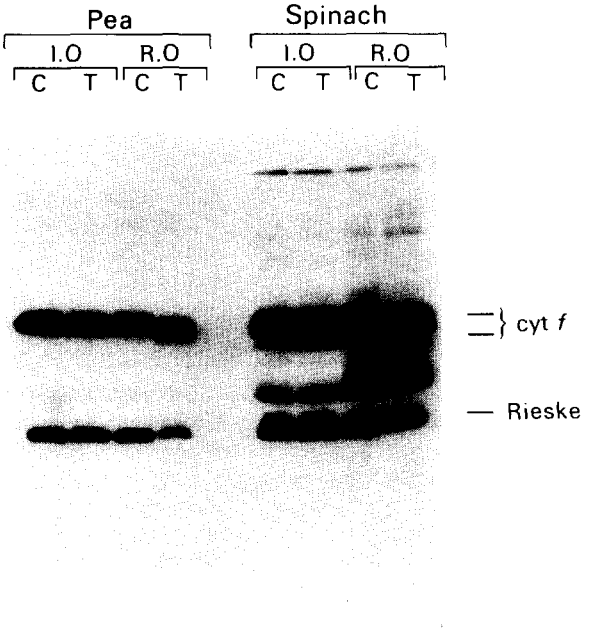

Fig. 5. The effect carboxypeptidase digestion on cytochrome *f* and the Rieske iron-sulphur protein from both pea and spinach thylakoids. Control (C) and treated (T) samples of inside-out (I.O) and right-side-out (R.O) vesicles were prepared as described in Fig. 3. Membranes were then electrophoretically separated under denaturing conditions and blotted onto nitrocellulose paper before incubation with anti-cytochrome *b*-563/*f* complex antiserum and ^{125}I-labelled protein A. The autoradiograph was exposed for 48 h at 193 K.

similarly carboxypeptidase-treated and electroblotted samples from pea leaves. The treatment led to the loss of an M_r-2000 fraction from the outer

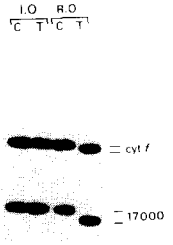

Fig. 6. The effect of carboxypeptidase on inside-out (I.O) and right-side-out (R.O) vesicles derived from pea thylakoids. Control (C) and treated (T) samples were analysed as in Fig. 5 but incubated with anti-M_r 17000 protein antiserum.

surface only. The intensity of the autoradiograph band did not decrease significantly, which suggests that the lost fraction was not highly antigenic. The antibody used in the experiment shown in Fig. 6, was raised against the M_r 17000 protein which had been eluted from narrow gel slices. However, there was extensive reaction with a protein of M_r 34000, probably cytochrome *f*. This was unlikely to be due to cross-reactivity of the two, since, in a sequence homology test, we found little similarity between cytochrome *f* and the M_r 17000 protein (using the sequences detailed in Refs. 7 and 10). The effect is probably a result of co-migration of breakdown products of cytochrome *f* with the test protein on the original gel.

It should be noted that all four proteins studied here have been found to be susceptible to carboxypeptidase treatment at the outer membrane surface. We were unable to discern the location of the amino-terminal ends, since the aminopeptidase used did not affect either the antigenicity or the mobility of any of the proteins of the cytochrome complex. Such a negative result is inconclusive, since the use of proteinases to investigate membrane topography is only effective in cases where susceptible sites are exposed. It is possible for a protein to protrude from a membrane but be unaffected by a proteinase in the absence of such sites.

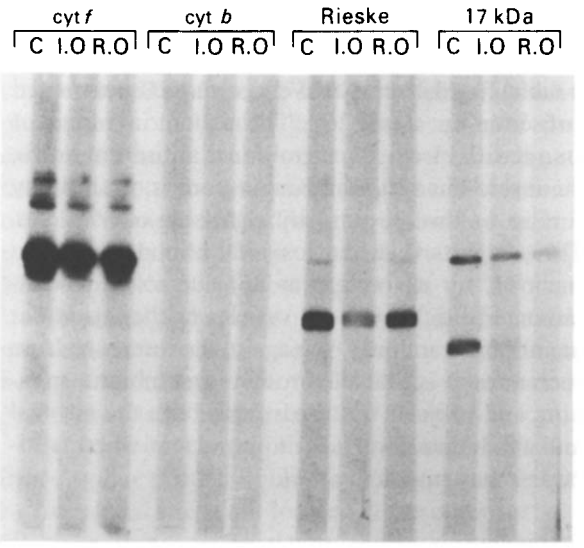

Fig. 7. Adsorbtion of antisera raised against specific proteins by inside-out (I.O) or right-side-out (R.O) vesicles prepared from pea thylakoids. Samples of membranes were added to aliquots of antisera to give 0.25 mg Chl : 20 μl antiserum. All samples were then made to 1 ml with a solution containing 0.9% NaCl, 10 mM Tris-HCl (pH 7.4) and 3% bovine serum albumin. Samples were gently shaken for 30 min at 25°C. Membranes were removed from suspension by centrifugation (35000 × *g*, 90 min) and the extracted antisera were incubated with individual strips of nitrocellulose sheet upon which cytochrome complex had been blotted from denaturing SDS gels (10 μg protein/track). C, control or unextracted antiserum.

Antibody adsorbtion studies

In order further to probe protein exposure at the membrane surfaces we determined whether the membrane vesicles adsorbed antibodies from test antisera. Inside-out and right-side-out vesicles were mixed, under controlled conditions, with antisera raised against individual polypeptides from the cytochrome complex. Depleted antisera were then reacted against standard amounts of electrophoretically separated and blotted cytochrome *b*-563/*f* complex. The amounts of antibody remaining in these depleted antisera were then compared with unextracted samples (see Fig. 7). Antibodies raised against cytochrome *f* were depleted from the antiserum by both inside-out and right-side-out vesicles (compare Fig. 7, track 1 with tracks 2 and 3). However, at the membrane concentration used here there was no clear difference in the extent of binding to either surface. There was no reaction against cytochrome *b*-563, even with the unextracted antiserum (Fig. 7, track 4). A band, corresponding to the cytochrome, was present on the nitrocellulose blot (as detected by staining with amido black, not shown) so it is probable that no antibody was raised against this protein. Antibodies were successfully raised against the Rieske protein and the M_r 17000 protein (Fig. 7, tracks 7 and 10 respectively). The anti-Rieske protein antiserum was contaminated with antibodies to cytochrome *f*, probably for the same reason discussed earlier for the contamination of the anti-M_r 17000 serum. It is clear that both inside-out and right-side-out vesicles adsorbed antibodies to the Rieske protein from the antiserum. Binding was more efficient at the inner surface (compare tracks 8 and 9, Fig. 7). Thus, the Rieske protein is shown to be membrane-spanning, yet with a major antigenic fraction exposed in the intrathylakoid lumen. Sim-

ilarly, both inside-out and right-side-out vesicles bound antibodies to the M_r 17000 protein (Fig. 7, tracks 11 and 12 respectively, with the unextracted antiserum in track 10). Throughout a range of assays with increasing membrane : antiserum ratios, the inside-out vesicles bound amounts of antibody similar to those bound by right-side-out vesicles. This suggests that the loss of antibodies seen in track 11, for example, was not due solely to contaminating right-side-out vesicles in the inside-out membrane fraction. Once again, the inference from such a result is that the protein is membrane-spanning and projects outwards from both the stromal and the lumenal surfaces to an extent where antibodies may interact with it.

Discussion

The chloroplast cytochrome complex facilitates transmembrane electron flow and also interacts with electron-transporting proteins at both the inner and the outer surfaces. Therefore, the complex as a whole presumably spans the membrane and has different functional groups exposed at each side. The best membrane fractions currently available to aid study of this organization are the oppositely oriented thylakoid vesicles prepared by phase partition in aqueous polymers [6]. Other membrane fractions do not have well-defined orientation or, for those that do, the cytochrome complex is missing. One disadvantage of vesicles separated by phase partition is that cross-contamination of membrane types occurs (30% right-side-out in the inside-out fraction and 11% inside-out in the right-side-out fraction [6]). An apparent loss of protein staining or antibody interaction upon proteolysis of one membrane fraction might be due to an effect on the contaminating material only. The results presented here, therefore, are not absolutely quantitative. However, it was possible to determine whether various proteins were more or less accessible to proteinases or antibodies at one or other membrane surface.

Cytochrome *f* has been shown previously, in studies involving proteinases, to have an exposed fragment at the inner surface [7,8] and to have its carboxyl-terminal end exposed to the stromal matrix [7]. These results are confirmed here by both proteolytic attack and antibody adsorption experiments. As noted by Hurt and Hauska [23], there appears to be a heterogeneous population of cytochrome *f*, with apparent M_r values of 34000 and 33000. This was also found in the present study (Figs. 3 and 5) and it is clear from Fig. 5 that carboxypeptidase has an equivalent effect on both cytochrome *f* proteins, i.e., the major and minor bands each lose about M_r 1000 upon treatment. Since both cytochrome *f* fractions respond in the same way to carboxypeptidase it is likely that their stroma-exposed carboxyl regions are identical. Therefore, the difference between the two populations of cytochrome *f* may be located on that section of the protein located within the intrathylakoid lumen. From amino acid sequence data, Willey et al. [7] suggested that nearly 90% of the cytochrome protein is located within the lumen, and that a total of 26 arginyl and lysyl peptide bonds are to be found in this region. These bonds are trypsin-labile and, if the above model is correct, must be largely inaccessible to the proteinase, since we have found only a limited effect of trypsin on cytochrome *f* in inside-out vesicles. However, although both inside-out and right-side-out vesicles adsorbed antibodies from an antiserum raised against cytochrome *f*, there was little difference in the extent of adsorbtion (Fig. 7). Our data indicate the presence of an equal distribution of antigenic sites at each membrane surface. This does not preclude the possibility of an extreme asymmetric arrangement of cytochrome *f* across the membrane, as put forward in Ref. 7. However, we suggest that further biochemical studies need to be carried out to ascertain the validity of the proposed model of cytochrome *f* [7].

We were unable to obtain immunological data concerning the location of cytochrome *b*-563, since no antibodies could be raised against the protein with either the isolated cytochrome complex or purified protein as the antigen. However, we were able to obtain information from studies using the haem-associated peroxidase technique, in conjunction with mildly denaturing conditions during electrophoresis. Cytochrome *b*-563 was most affected by trypsin when the inner surface was exposed (Figs. 1 and 2), although some loss of haem-binding occurred during digestion at the outer surface. Moreover, carboxypeptidase most effectively disrupted haem-binding by cytochrome

b-563 when the outer surface was exposed. It is clear that the cytochrome is susceptible to proteinases at both surfaces. This finding is compatible with the molecular model proposed by Widger et al. [10] in which parts of the protein extend out on either side of the membrane. Their model indicates that there may be trypsin-susceptible residues on most of the exposed sections so that proteolytic digestion would result in the production of numerous small fractions. Such fractions would probably no longer retain any haem-binding capacity, hence our observation of a progressive decrease in 'haem-stain' intensity upon trypsin treatment (Fig. 1). Moreover, the loss of mass (approx. M_r 1000) by cytochrome *b*-563 following tryptic digestion at the inner surface (Fig. 1) is consistent with the proposed location of one lysine and four arginine residues 5, 9, 11, 12 and 14 amino acids distant, respectively, from the amino terminus [10].

From the results of immuno-aggregation studies, Morschel and Staehelin [9] suggested that cytochrome *f* and possibly cytochrome *b*-563 are exposed at the inner surface, but that no antigenic determinants are present on the stromal side of the membrane. In this study we have detected antibody interaction with cytochrome *f* at the outer surface (as also reported in Ref. 24) as well as proteinase interaction with cytochrome *b*-563 at the same surface. The 'haem-staining' and antibody absorption techniques used here are probably more sensitive than the agglutination method used in Ref. 9.

Widger et al. [10] proposed that the M_r 17000 component has an amino acid sequence similar to the carboxyl end of the mitochondrial *b*-type cytochrome and that the two may, therefore, be equivalent. The structural model put forward for this region has four α-helical membrane-spanning groups and five regions of the protein which extend out of the membrane [10]. We have shown here (Fig. 7) that antibodies raised against the M_r 17000 protein interact with both sides of the membrane, indicating that the protein is membrane-spanning and projects into both the stroma and the intrathylakoid lumen, features consistent with the above model. The carboxyl terminus of the M_r 17000 protein was found to be located at the outer surface (Fig. 6), as was demonstrated also for cytochrome *b*-563. If the molecular model for the *b*-type cytochrome [10] is fully applicable to cytochrome *b*-563 and the M_r 17000 protein of chloroplasts, then our knowledge of the location of the carboxyl termini enables us to hypothesize about the position of the amino termini. The N-terminus of cytochrome *b*-563 is likely to be located within the intrathylakoid lumen, whereas that of the M_r 17000 protein would be located in the stroma. Unfortunately, the aminopeptidase used here did not interact with the proteins. This may have been because of its large size (300 kDa); other amino proteinases may be more useful.

Evidence concerning the location of the Rieske iron-sulphur protein in chloroplasts suggested that its functional site is not located at the outer surface (electron transport in whole thylakoids was not inhibited by a specific antibody [12]). However, we show here that antibodies do bind to the protein at this surface (Fig. 7) and also that the carboxyl terminus is exposed to the stroma (Fig. 5). The most efficient antibody binding to the Rieske protein occurred with inside-out vesicles (Fig. 7). Therefore, we suggest that the major antigenic region of the protein is located at the inner surface. This finding clarifies the evidence put forward by Hurt et al. [12], who showed that specific antibodies interact with detergent-isolated cytochrome complex. Their results do not clearly distinguish whether the antigenic determinants are located within or on the surface of the membrane. Our results concerning the location of the Rieske protein correspond well with the analogous location of this protein near the cytochrome c_2 side of *R. sphaeroides* photosynthetic membranes [11].

In summary, we have demonstrated that cytochrome *f*, cytochrome *b*-563, the Rieske iron-sulphur protein and the M_r 17000 component of the chloroplast cytochrome complex are all transmembrane proteins. All have their carboxyl terminus exposed to the stromal matrix. This may be significant with respect to the mechanisms by which the proteins are inserted into thylakoid membranes and are anchored into functional positions. Our results lend biochemical support to the recently proposed models of cytochrome *f* [7], the *b*-type cytochrome and the M_r 17000 protein [10]. These models are based, in part, on analyses of the relative hydrophobicity of sections of the ap-

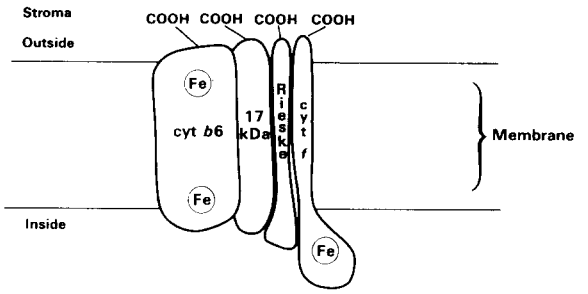

Fig. 8. A schematic diagram indicating the two-dimensional organisation of the polypeptides of the chloroplast cytochrome *b*-563/*f* complex across the thylakoid membrane.

propriate amino acid sequences. A chain of about 20 hydrophobic amino acids will enable that section of a protein to span the thylakoid membrane in an α-helical configuration. However, such a hydrophobic sequence might also be sequestered within the protein, external to the membrane. Moreover, shorter hydrophobic sections may cross the membrane in paired β-pleated sheets [25]. Additional evidence, such as that provided here, is necessary to substantiate these models. Our findings support the previously proposed orientation of cytochrome *f*, but do not indicate the presence of an extensive moiety at the inner surface (c.f. [7]). On the other hand, the data do support the proposed organization of cytochrome *b*-563 and the M_4 17000 protein [10]. In neither case, however, are we able to derive the protein configuration within the membrane.

Our results do not define the location of functional groups involved in electron transfer. However, it is clear that the transmembrane arrangement of the cytochrome complex, as demonstrated here, is conducive to vectorial electron flow. Moreover, the location of major parts of both the Rieske protein and cytochrome *f* at the inner surface may be important for plastoquinol oxidation and plastocyanin reduction at that surface. We have summarized our results in a schematic diagram (Fig. 8) which is intended to outline the transmembrane organization within the cytochrome complex. No three-dimensional structure for the cytochrome *b*-563/*f* complex can be suggested at this point, since crystallographic studies of the whole or subdivided complex are not yet available.

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