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ETUDE DES PROTEINES RADIOACTIVES ASSOCIEES A L'ENVELOPPE DU CHLOROPLASTE DE L'EPINARD APRES PROTEOSYNTHESE *IN ORGANELLO* EN PRESENCE DE ^{35}S-METHIONINE

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PAR NICOLE DUMONT-BEBOUX

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Etude des protéines radioactives associées à l'enveloppe du chloroplaste de l'épinard après protéosynthèse in organello en présence de ^{35}S-méthionine

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Characteristics of Spinach Chloroplast Envelope, Thylakoid and Stroma Polypeptides as Revealed by Triton X-114 Phase Partition

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Comparison of the SDS-PAGE profiles of the spinach chloroplast stroma, thylakoid and envelope membranes shows that several polypeptides have the same electrophoretic mobility. To simplify these somewhat complex electrophoretic profiles and to verify whether the polypeptides having similar electrophoretic mobility are identical, we used Triton X-114 phase partition to obtain a separation of the polypeptides according to their relative hydrophobicity. The stroma polypeptides partitioned essentially in the aqueous phase. About half of the thylakoid and envelope membrane polypeptides were exclusively recovered in either one of the two phases. Therefore, the phase partitioning of membrane polypeptides proved to be useful, as the organic phase contained true intrinsic polypeptides, while the aqueous phase was composed of peripheral ones and stroma components. Particularly interesting was the release of the RubisCO large subunit known to copurify with the envelope membranes. Additional experimental approaches were used (immunology, proteosynthesis *in organello*) to further characterize proteins which had apparent ambiguous phase partitioning. Here, we show that Triton X-114 is an excellent tool to unmask polypeptides having identical electrophoretic mobility but different behaviour towards this detergent; its use leads to a clarification of the polypeptide SDS-PAGE profiles of chloroplast membranes.

Key words: Electrophoretic separation — Envelope membranes — Hydrophobicity — Immunology — Spinach chloroplast polypeptides — Triton X-114.

Chloroplasts from higher plants are made up of six different compartments (the outer and inner envelope membranes, the intermembrane space, the stroma, the thylakoid membranes and the thylakoid lumen), each of which having its own functions and a distinct set of polypeptides. The envelope serves as a boundary between the cytosol and the chloroplast and is the site of many enzymatic activities on top of its role as a selective barrier for the movement of molecules in and out of the chloroplast (Douce et al. 1984). The envelope, the polypeptides of which represent only 1–2% of the total chloroplast proteins, is composed of two membranes with different densities and permeabilities (Douce et al. 1984).

Abbreviations: Mops, (3-[*N*-morpholino]propanesulfonic acid); PMSF, phenylmethane sulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; IEF, isoelectric focusing; pI, isoelectric point; TX-114, Triton X-114 (alkylphenylpolyethyleneglycol); TX-100, Triton X-100; T, thylakoid; S, stroma; IM, inner membrane; OM, outer membrane; RubisCO, ribulose 1,5-bisphosphate carboxylase/oxygenase; Mr, apparent molecular weight.

The electrophoretic analysis of the inner and outer envelope membranes in one-dimension shows that each membrane has its own set of polypeptides which differ from those of the thylakoid and the stroma (Joyard et al. 1982, Werner-Washburne et al. 1983). However, the two envelope membranes are linked together by contact sites as shown by freeze-fracture electron microscopy (Cline et al. 1985) and freeze-fracture and freeze-substitution combined with ultrathin sectioning (Cremers et al. 1988). Upon separation of the two membranes in hypertonic medium followed by their purification, these contact sites give rise to stroma-containing vesicles migrating with the inner membrane during centrifugation. The characterization of the two envelope membranes is thus complicated by the presence of stroma. Furthermore, even though the envelope membrane purification methods have been greatly improved, it is not possible to obtain pure fractions and the electrophoretic profiles may not be totally representative of the subchloroplast fractions studied.

Moreover, they are difficult to analyze as different polypeptides may display the same electrophoretic mobility and

migrate as a unique Coomassie blue band.

In an attempt to go deeper into the characterization of the two envelope membrane polypeptides and to assess and identify the source of any cross-contamination that may occur during the purification of the two membranes, we have used TX-114 temperature-induced phase partition as described by Bordier (1981). TX-114 is the only detergent of the TX-100 series which forms clear micellar solutions in water at low temperature (4°C) and separates above 20°C into two phases, respectively enriched and depleted in detergent. The TX-114 phase partition has been used successfully by Bricker and Sherman (1982) on maize thylakoid membranes and, more recently, by Kjellbom et al. (1989) on spinach leaf plasma membrane. Here, we have applied the TX-114 phase partition method to chloroplast envelope membranes with the specific aim of clarifying their complex electrophoretic profiles. For comparison, we have included stroma and thylakoid membrane electrophoretic separations in order to better detect possible contamination by these fractions. This technique gives rise to a preliminary separation of the proteins according to their hydrophobic/hydrophilic character while keeping them in a membrane-like environment, thus preserving their native form. This treatment, followed by electrophoresis, enables to separate several polypeptides of similar Mr but of different behaviour towards TX-114 phase partition and helps to identify polypeptides of different origin. However, due to ambiguous phase partitioning and to polypeptide comigration that may still exist, it was imperative to include other criteria based on results from 2-D electrophoresis, immunology and proteosynthesis *in organello*, to differentiate between those polypeptides that could have been otherwise mistaken for others. The present study extends the results already presented in 1988 (Dumont et al.) and 1989 (Dumont and Siegenthaler).

Materials and Methods

Isolation of purified, intact chloroplasts—Chloroplasts were obtained from 800 g of spinach leaves bought at the local market. The deribbed leaves were blended in a four-liter Waring blender for a total of 10 to 15 sec in a chilled grinding medium (250 ml/100 g of leaves) containing 350 mM sorbitol, 25 mM Mops, 2 mM EDTA-Na_2 and 2 mM isoascorbate adjusted to pH 7.6 with KOH. After filtration on 6 layers of muslin and one layer of cheesecloth, the resulting filtrate was centrifuged at 2,100 × *g* (Sorvall GSA rotor) for 1 min. Each pellet was resuspended in 10 ml of grinding medium and layered on top of a 40% Percoll solution containing the same ingredients as the grinding medium. After centrifugation (2,100 × *g* in a Sorvall HB-4 rotor for 3 min), the pellets of purified chloroplasts were resuspended in the grinding medium and centrifuged at 2,000 × *g* (Sorvall SS-34 rotor) for 3 min to wash out the residual Percoll.

Purification of envelope membranes—The fractionation of the chloroplasts into soluble and membrane proteins was achieved mainly according to Keegstra and Yousif (1986). The purified chloroplasts were resuspended in hypertonic medium and the chlorophyll adjusted at 2.5 mg per ml (Bruinsma 1961). After a 15 min incubation on ice, the chloroplasts were ruptured with a Dounce homogenizer and diluted with 2 vol of TE buffer (10 mM Tricine-NaOH, pH 7.6, 2 mM EDTA-Na_2). The thylakoids were sedimented by a three 10 min steps differential centrifugation at 4,500, 13,500 and 23,500 × *g* in a Sorvall HB-4 rotor using slow acceleration. This centrifugation procedure led to a substantial increase in the envelope membrane yield. The yellow supernatant was layered on top of a discontinuous sucrose gradient consisting of 8 ml of each of 1 M, 0.65 M and 0.4 M sucrose solutions buffered with TE + $MgCl_2$ (5 mM) in the presence of 1 mM PMSF and centrifuged for 6 h at 100,000 × *g* in a Beckman SW-27 rotor. The stroma was recovered at the top of the gradient. The outer and inner envelope membrane fractions, found at the sucrose interfaces, were collected, diluted with TE + PMSF and sedimented at 100,000 × *g* for 60 min in a SW-27 rotor. After resuspension in TE + PMSF, the protein content was measured (Bradford 1976).

Separation of stroma, thylakoid and envelope membrane proteins into hydrophobic and hydrophilic fractions—The proteins of the stroma, the thylakoid and the inner and outer envelope membranes were separated according to their hydrophobicity and hydrophilicity using the properties of Triton X-114 as described by Bordier (1981). The original method was slightly modified: in order to avoid excessive loss of proteins, no sucrose cushion was used between the aqueous and the detergent phases; the detergent phase was extracted twice and its surface washed to eliminate contamination from the soluble proteins. The protein sample (100 μg) was adjusted to 1% TX-114 with a solution containing 2% of precondensed TX-114, 10 mM Tris-HCl, pH 7.4 and 150 mM NaCl and solubilized for 10 min at 0°C. To allow condensation, the sample was then incubated for 10 min at 30°C in a water bath before being centrifuged for 3 min at 300 × *g* in a swinging bucket rotor (Universal Hettich 1200) at room temperature. From then on, the two phases were treated separately and the extraction was repeated once for the detergent phase and twice for the aqueous one. The surface of the detergent phase was washed with 500 μl of buffer. The proteins of the two phases of interest were precipitated overnight in ice cold 80% acetone at −20°C. They were then centrifuged for 10 min at 10,500 × *g* in a Sorvall HB-4 rotor before being prepared for isoelectric focusing or SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

Electrophoresis and autoradiography—The precipitated proteins were denatured in boiling water for 3 min in

2% SDS, 0.005% bromophenol blue (w/v), 6.25% glycerol and 5% mercaptoethanol (v/v) in 0.0625 M Tris-HCl, pH 6.8. The samples were electrophoresed essentially according to Laemmli (1970) for 5 to 6 h on slab gels containing either 12% acrylamide throughout or a linear 10 to 18% (w/v) acrylamide gradient accompanied by a 5 to 15% (w/v) sucrose gradient. The acrylamide to *N,N'*-methylenebisacrylamide ratio was 30 : 0.8. The gels were fixed in a destaining solution containing 25% denatured ethanol and 8% acetic acid. They were either stained in 0.25% Coomassie brilliant blue R-250 (w/v) in 50% methanol and 10% acetic acid (v/v) or as described by Neuhoff (1988). After destaining, they were dried under vacuum. Prior to drying, the bands of the 10 to 18% gradient acrylamide gel were scanned at 600 nm in a Zeiss-Disc ZK4 gel scanner and apparent mol wt estimated by calculating the relative mobility of the polypeptides compared to the following calibration proteins: phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa). Autoradiographs were prepared on Kodak X-Omat AR films.

Isoelectric focusing—The organic and aqueous samples, obtained from an initial amount of 300 μg of protein, were solubilized as described by Dunbar (1987). The isoelectric focusing procedure and pH determination were essentially according to Siegenthaler and Nguyen (1983). The gels were 7 cm long and contained 2% (v/v) TX-100 instead of Nonidet-P40. At the end of the run, the gels were treated as described by Jäckle (1979) before being submitted to the second dimension on a 12% SDS-gel.

Western blotting—After SDS-PAGE, the polypeptides were transferred to cellulose nitrate sheets using a semi-dry electroblotting apparatus (Sartorius). The electrophoretic buffer contained 48 mM Tris, 39 mM glycine, 1.3 mM SDS and 20% (v/v) methanol (pH at about 9) (Heegaard and Bjerrum 1986).

Immunological studies—The control and TX-114 organic and aqueous phase fractions of the thylakoid, stroma and envelope polypeptides were challenged with several antibodies by the method of Towbin et al. (1979), except that 10 and 3% bovine serum albumin were replaced by 5 and 1% powdered milk and that no carrier serum was used. The secondary antibody directed against the first antiserum was labelled with alkaline phosphatase (KPL Laboratories) and used as described by Leary et al. (1983). Antibodies raised against the ribulose 1,5-bisphosphate carboxylase/oxygenase were kindly provided by Dr. A. Radunz.

Results and Discussion

Although the electrophoretic patterns of the four purified chloroplast fractions, stroma (S), thylakoids (T), inner (IM) and outer (OM) envelope membranes, are different, there are some comigrating polypeptides which may be either polypeptides with identical Mr but different physicochemical properties or the result of some cross-contamination. Furthermore, the stroma can be expected to contain polypeptides of nucleic origin which accumulate there when their insertion into the thylakoid membranes is prevented (Cline et al. 1989). Polypeptides of chloroplastic origin can also be expected to accumulate there.

To investigate these possibilities, we separated the hydrophobic from the hydrophilic polypeptides in each of these fractions, using the Triton X-114 phase partition method.

For sake of clarity, it is necessary to remind (Bennett 1982) that membrane proteins have been classified as integral or peripheral proteins. Integral membrane proteins can be defined as being "globular" or "fibrous". The "globular" proteins, such as a membrane-spanning protein, have a significant portion of their mass within the bilayer, while the "fibrous" ones have most of their mass protuding from the membrane and a hydrophobic polypeptide tail dipping into the membrane bilayer. Peripheral proteins, on the other hand, are membrane bound through non-covalent, probably mainly electrostatic interactions with the hydrophilic regions of integral proteins or with headgroup regions of the lipid bilayer. Once separated from the membrane, these proteins behave as soluble proteins and will therefore be recovered in the detergent-free phase.

From the definition given above, it is expected that "globular" proteins will partition in the detergent phase, while the "fibrous" ones, depending on the relative importance of their hydrophobic tail, may have an ambiguous behaviour and be recovered in the two phases.

TX-114 phase partition: extraction conditions

In his original procedure, Bordier (1981) recommended one extraction of the organic phase and three for the hydrophilic one. However, preliminary results showed that two extractions of the detergent phase were necessary to achieve a complete removal of the hydrophilic polypeptides.

SDS-PAGE analysis of the four TX-114-treated fractions

Introduction—Four fractions (T, S, IM and OM) were prepared and then submitted to TX-114 extraction. The electrophoretic patterns of the resulting aqueous and organic phase polypeptides, along with their corresponding controls, are shown in Fig. 1. Although each phase contained its own set of polypeptides, there were still several similarities in the electrophoretic mobilities. Among the polypeptides having the same electrophoretic mobility, some had a similar behaviour towards TX-114 treatment and might therefore be identical, while others did not partition in the same phase, thus indicating their different

nature. As no chlorophyll could be detected in the stroma or the envelope membranes, contamination by thylakoid components could be ruled out. However, stroma material, arising from inner membrane-derived vesicles, can be present in the envelope fractions (Cremers et al. 1988) as well as in the thylakoid one.

Membrane phase partition—The complexity of the electrophoretic patterns of the membrane polypeptides, especially those of the envelope membranes (IMc, OMc), can be partly overcome by separating these polypeptides according to their hydrophobic and hydrophilic properties. For instance, one can see (Fig. 1, IMo, OMa) that most of the inner membrane polypeptides were recovered in the organic phase while those of the outer membrane partitioned preferentially in the aqueous phase. On close examination, about half of the envelope membrane polypeptides were recovered in only one of the two phases. The partitioning of the polypeptides was therefore quite good whenever strongly hydrophobic or strongly hydrophilic polypeptides were concerned. Indeed, the inner membrane 94, 34 and 33 kDa, the outer membrane 76, 66 and 22 kDa polypeptides, the thylakoid LHCP and Cyt. f (as localized also by Bricker and Sherman 1982) as well as several thylakoid low Mr polypeptides were exclusively recovered in the organic phases (Fig. 1, IMo, OMo and To). In contrast, the thylakoid 30 kDa and the outer membrane 109, 40, 24, 23 and 21 kDa polypeptides (Fig. 1, Ta and OMa) were only encountered in the aqueous phase. Consequently, by discriminating between hydrophobic and hydrophilic polypeptides, TX-114 enhanced the SDS-PAGE resolution.

Other polypeptides were common to the two phases and their characterization as integral ("globular" and "fibrous") or peripheral is more difficult. As there are several answers to that situation, complementary methods have to be used. Here, we may be dealing with polypeptides that were not totally removed from the membrane (Bordier 1981). These polypeptides could be "fibrous" proteins, the solubilization of which depends strongly on the nature of their hydrophobic tail and its interactions with the membrane. Such may be the case of the 1M81 and 70 kDa polypeptides which were found simultaneously in both the organic and aqueous phases, as revealed by 2D electrophoresis (see arrows in Fig. 2A and B). However, the α and β subunits of CF_1, known as being hydrophilic polypeptides, failed to partition completely in the aqueous phase (see Figs. 1 and 3, To, a). As suggested by Bricker and Sherman (1982), a small amount of CF_1 may remain attached to CF_0 or, as these polypeptides are very abundant, there could be a spilling over phenomenon. In other instances, we were dealing with two or more totally different polypeptides. Indeed, the thylakoid 15 kDa band (Fig. 1 and Fig. 3A, Tc, o, a) partitioned unequally between the two phases. Figure 3B shows that it was in fact resolved into a hydrophobic polypeptide synthesized in the chloroplast (as attested by its ^{35}S-labelling in the organic phase) and a hydrophilic one of cytoplasmic origin (no labelling in the aqueous phase).

Comigration may also occur in one of the phases. As shown in Fig. 2C, a unique Coomassie band (Control: aqueous phase 72 kDa) may contain two polypeptides having different pI (see arrows). In this case, they were not charge isomers but again polypeptides of different genomic origin as shown by proteosynthesis experiments (results not shown).

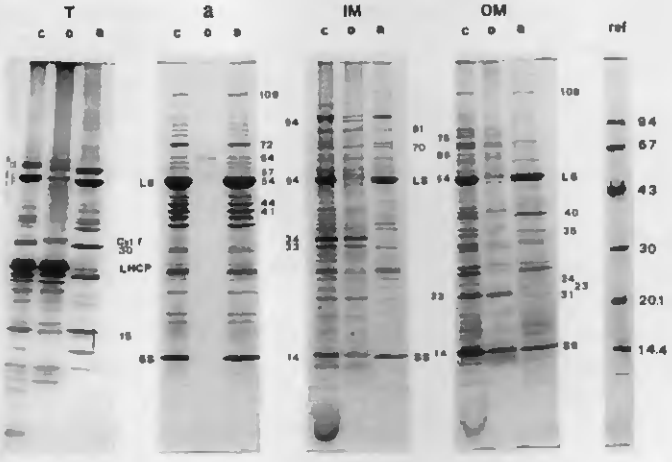
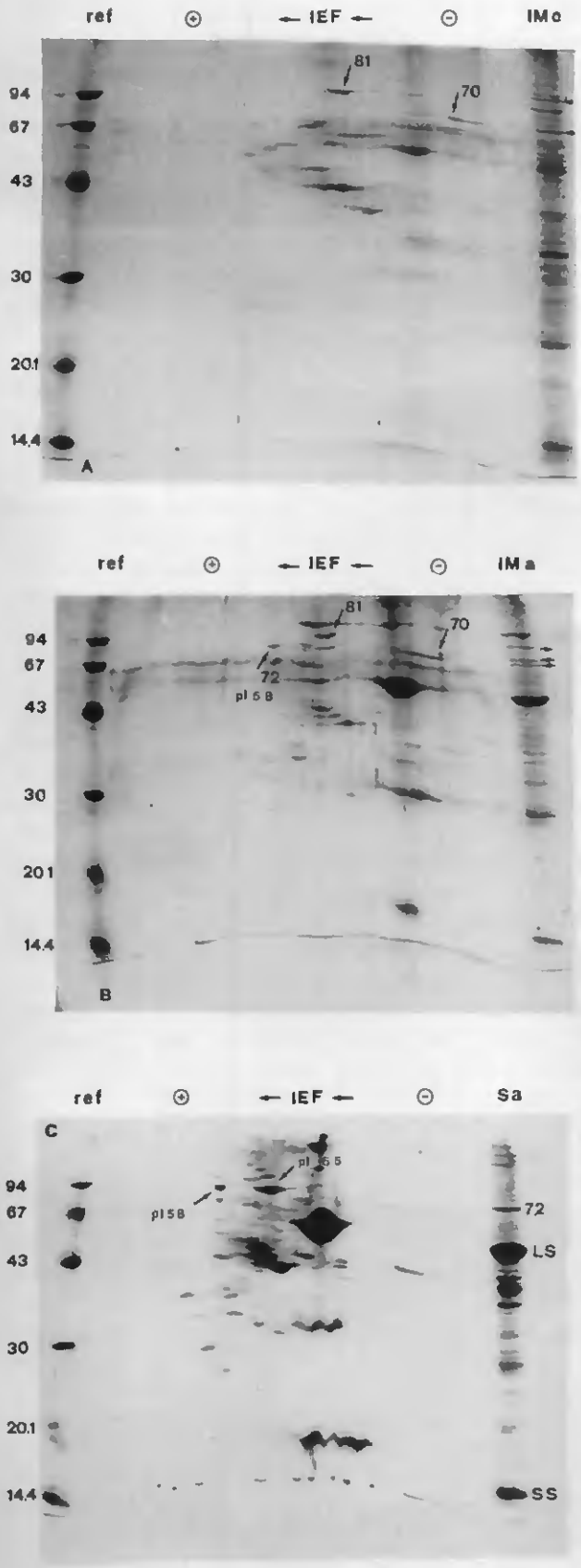


Fig. 1 Electrophoretic separation of thylakoid, stroma, inner and outer envelope membrane polypeptides. The TX-114 phases were extracted twice and the aqueous phases three times. The references (ref) and the separating gel containing a linear 10–18% acrylamide gradient are as described in Materials and Methods. Each control fraction (c) contained 30 μg of untreated polypeptides. The organic (o) and aqueous (a) fractions were obtained from an initial amount of 100 μg of proteins. T, thylakoid; S, stroma; IM, inner membrane; OM, outer membrane. Apparent mol wt values are indicated in kDa.



Stroma phase partition—All but five of the stroma polypeptides were recovered in the aqueous phase (Fig. 1 Sa). The presence of Coomassie bands in the organic phase (Fig. 1 So) was rather unexpected, but nevertheless, TX-114 phase partitioning was highly reproducible. These faint bands corresponded to polypeptides having Mr of 64, 57, 54, 44 and 41 kDa as determined by densitometric tracings (results not shown). Since bands of equivalent Mr could be seen in the organic phases of the membrane fractions, envelope and/or thylakoid components might have contaminated the stroma. But, if contamination by membrane material occurred, one would have expected to find all or at least most of the membrane hydrophobic polypeptides in the stroma organic phase (Fig. 1 So). Since it was obviously not the case, one can reasonably exclude any significant contamination of the stroma fraction by the envelope or thylakoid membranes. Furthermore, the stroma was unlikely to be contaminated by thylakoids since the stromal fraction did not contain any trace of chlorophyll. The 63 kDa band was doubtful as it comigrated with an artifact sometimes present in our electrophoreses. The presence of these hydrophobic proteins is best explained by assuming that we are dealing with transit polypeptides of chloroplastic (S57, 54 kDa) or nuclear (S44, 41 kDa) origin, as suggested by proteosynthesis experiments (results not shown) and by Cline et al. (1989). Indeed, it is expected that these polypeptides, the fate of which is to be eventually integrated in a membrane, contain several hydrophobic domains which would favour an organic phase-partitioning.

Peripheral membrane proteins or soluble contaminants?—Another interesting feature of the polypeptide separation in the presence of TX-114 was the unexpectedly great number of envelope membrane polypeptides having a hydrophilic character (Fig. 1 IMa, OMa). These may be peripheral (loosely membrane-bound) polypeptides or stroma components. The latter was indeed the case with the large and small subunits of the RubisCO which are known to copurify with the envelope membranes (Pineau et al. 1979, Joyard et al. 1982). Such was also the case with the inner membrane 72 kDa (Fig. 2B: pI 5.8) which, when present, reacted with the antibody against the stroma 72 kDa raised in our laboratory (data not shown). On the other hand, OM 35, 23 and 21, found exclusively in the membrane aqueous phase, did not have any counterpart in the stroma (Fig. 1 Sa). This strongly suggests that these outer membrane polypeptides are true peripheral com-

Fig. 2 2-Dimension electrophoretic separation of the organic (A) and the aqueous (B) phases of the inner membrane and the aqueous phase of the stroma (C). The IEF was as described in Materials and Methods and the separating gel of the second dimension contained 12% acrylamide.

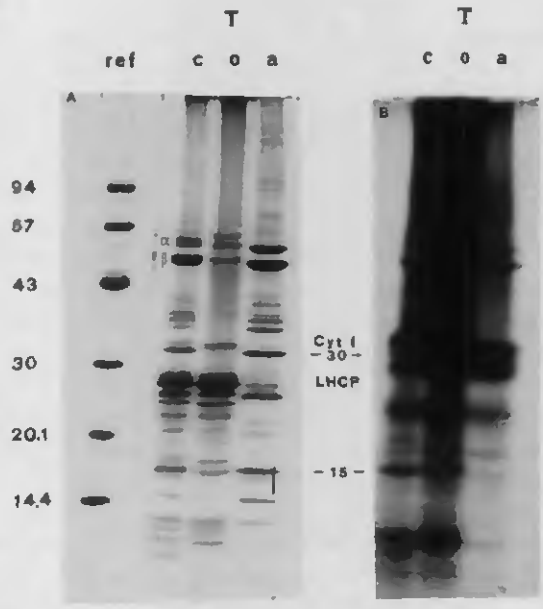


Fig. 3 Electrophoretic separation of labelled thylakoid polypeptides after TX-114 treatment (A) and its autoradiography (B). Experimental conditions and symbols as in Fig. 1. For proteosynthesis *in organello*, intact chloroplasts were resuspended (1 mg chl/ml) in an incubation medium containing 100 mM KCl, 2 mM $MgCl_2$ and 66 mM Tricine-KOH at pH 7.6. The light-driven incorporation of ^{35}S-methionine (150 μCi/mg chl; specific activity > 1,000 Ci/mmol; SJ 1515, Amersham) was performed in a water bath at 20°C for 60 min.

ponents of the envelope. It is thus clear that the preliminary separation obtained through the use of the TX-114 phase partition succeeds in eliminating all the stroma trapped in the inner or outer envelope membrane vesicles. The organic phase polypeptides are thus exclusively of membrane origin, while those found in the aqueous phase may still have a dual origin, namely membrane and stroma.

Proteins revealed by TX-114 phase partition—It is noteworthy that the use of this technique allowed the visualization of polypeptides which have close Mr and are normally partly overlapping. This was most obvious near the large subunit (LS) of the RubisCO. Indeed, a 54 kDa polypeptide was found in the organic phases of the stroma, of the inner and outer envelope membranes at the level of the RubisCO (Fig. 1 So, IMo, OMo). The RubisCO, in its 18 S holoenzyme form, is known to remain bound to the envelope membranes during their purification (Joyard et al. 1982) and has been, so far, impossible to eliminate (Pineau et al. 1979, Werner-Washburn et al. 1983). To assess the nature of this 54 kDa organic band, an antibody raised against the RubisCO was tested on the stroma, inner and outer envelope membrane TX-114-treated fractions (S, IM and OM) and their respective controls. The results are shown in Fig. 4. A positive reaction was found only in the controls and the aqueous phases of the three fractions. No reaction could be detected on any of the organic phases. Furthermore, a polyclonal antibody was raised in our laboratory against the stromal organic phase 54 kDa polypeptide. When tested, it did not react with LS in any of the aqueous phases but reacted with all three organic phases (see Fig. 5). The rather weak reaction of the controls can be explained by the presence of the LS which represents most of the 30 μg of total protein content. There is, therefore, a fair probability that the hydrophobic 54 kDa polypeptide, unmasked in the three fractions by TX-114 treatment, is not the large subunit. It is noteworthy that the RubisCO, which is not released by sonication and other drastic treatments (Joyard et al. 1982, Werner-Washburne et al. 1983), can be eliminated from the envelope membranes by TX-114 phase partition. Moreover, the RubisCO small subunit band in the IM and OM fractions was split between the organic and aqueous phases. Fig. 4 shows that the polypeptide with hydrophobic behaviour was not the small subunit (SS) as there was no immunological reaction with the RubisCO antibody.

Solubilization versus phase partition—The complex mixture of membrane proteins can also be fractionated by solubilizing them in chloroform/methanol (2 : 1 v/v) or in 0.1 N NaOH as described by Joyard et al. (1982). On the basis of their results, these authors concluded that 55 to 60% of the total envelope polypeptides are integral membrane proteins, most of them being characterized by a high Mr. A hydrophobic character was assigned to half of these integral proteins by virtue of their chloroform/methanol solubility. The other 40 to 45% of the envelope polypeptides were released from the membrane by NaOH treatment and were thus considered as peripheral proteins. The above two solubilization methods may lead, through delipidation or limited saponification of lipids, to a total or partial destruction of the membrane. Furthermore, the quaternary and tertiary conformations of the proteins are probably not preserved, leading to the exposure of hydrophilic and hydrophobic domains which may be erroneously determinant towards the final solubilization and the characterization as intrinsic or extrinsic. This drawback does not happen with TX-114 phase partition, which has been shown by the works of Sanchez-Ferrer (1989a, b, 1990) and Soll and Bennett (1988) to keep the proteins in their native state, thus allowing the isolation of functional enzymes. The resulting protein separations obtained by TX-114 solubilization and phase partitioning or by solubilization in chloroform/methanol or NaOH are in fact based on very different properties and cannot be compared. Indeed, the polypeptides E37 and E24 described by Joyard et al. (1982), corresponding respectively to our IM34 and OM22 (Fig. 1, IMo, OMo) were not solubilized in chloroform/methanol but partitioned in TX-114 as integral poly-

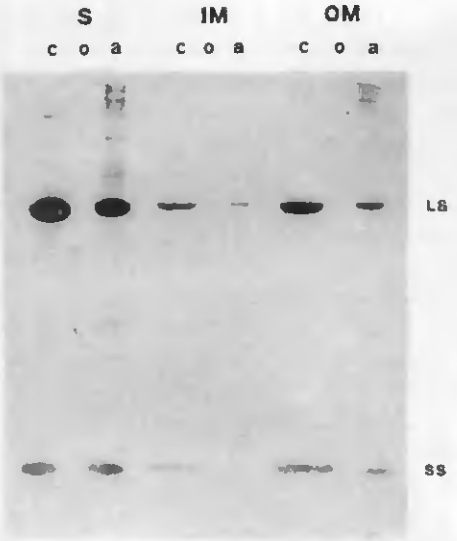


Fig. 4 Western blot of stroma, inner and outer envelope membrane polypeptides after treatment with TX-114 and separation on a 12% acrylamide gel slab. Antibodies against the RubisCO (LS, SS) were tested. Symbols are as in Fig. 1.

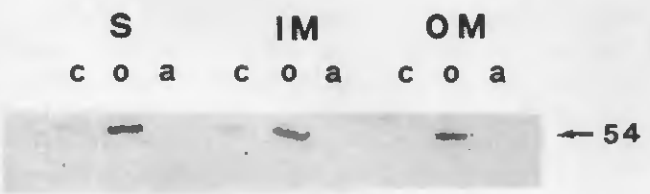


Fig. 5 Antibodies against the hydrophobic 54 kDa were tested on a Western blot as described in Fig. 4. Only the relevant part is shown. The antigen was purified on SDS gel electrophoresis. The desired gel bands were pooled, crushed in liquid nitrogen and dissolved in the complete Freund's adjuvant diluted 1 : 1 with distilled water. Three injections were made intradermically at 2 to 3 weeks intervals in a white New Zealand rabbit. Symbols are as in Fig. 1.

peptides. However, the two results taken together could very well indicate that E37 and E24 are "fibrous" proteins according to the definition given above. However, it is interesting that the E14 (corresponding to our IM14 and OM14) which was not totally extracted by chloroform/methanol (Joyard et al. 1982), was also found split between the aqueous and organic fractions of the inner and outer membranes (Fig. 1 IMa, o and OMa, o). As we have shown, theses organic bands were not related to the RubisCO small subunit as they did not react with the RubisCO antibody (Fig. 4). It is also likely that the faint band (at the E54 level), found by Joyard et al. (1982) in the chloroform/methanol-soluble fractions from stroma and envelope, is identical to the one found in our stroma and membrane organic fractions, and which was shown to be different from the RubisCO LS (Figs. 1, 4 and 5).

Conclusions—To decrease the complexity of the electrophoretic patterns of the envelope membrane polypeptides, it is thus advisable to fractionate the total set of proteins prior to electrophoresis. The TX-114 phase partition method, which is highly reproducible and easy to handle, has been shown here to be a valuable tool for chloroplast envelope membranes. It provides a physico-chemical environment suitable for membrane protein purification and allows the separation of integral proteins from peripheral and soluble ones.

Furthermore, the organic phases of the inner and outer envelope membranes contain "globular" proteins probably involved in chloroplast transport and communication. Moreover, the two RubisCO subunits, which are always present in the three membraneous fractions, cannot be dislodged from the two envelope membranes unless TX-114 phase partition is used. However they are easily washed out of the thylakoid. This suggests (as also proposed by Joyard et al. 1982) that the RubisCO could be specifically associated with the chloroplast envelope.

Therefore, the ability of TX-114 to separate membrane proteins according to their relative hydrophobicity while preserving their native form is a potentially very powerful method towards the characterization and purification of envelope chloroplast polypeptides.

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Technical Article

Optimization of an incubation medium specific for protein synthesis in isolated intact spinach chloroplasts

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We present here an incubation medium specially devised for light-driven protein synthesis in intact spinach (*Spinacia oleracea* L., var. Nobel) chloroplasts. The incorporation of [^{35}S]methionine was optimal when the assay was performed at 20°C in a medium containing 66 mM Tricine-KOH (pH 7.6), 100 mM KCl, 2 mM $MgCl_2$ and 50 μM cold amino acids. ATP was found to be slightly inhibitory. The total uptake (10% of the initial ^{35}S label) was distributed between the thylakoid (about 79%), the stroma (20%), the inner and outer envelope membrane proteins (0.8 and 0.2%). This incubation medium should be useful for other studies dealing with chloroplast translation products.

Additional key words – Thylakoid, stroma, chloroplast envelope, proteosynthesis, *In organello*.

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Abbreviations. a.a., amino acids; ATP, adenosine triphosphate; CAP, D-*threo* chloramphenicol; Chl, chlorophyll; CHX, cycloheximide; EDTA-Na_2, natrium salt of ethylenediaminetetraacetic acid; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis-(5-phenyl-2-oxazolyl)-benzol; TCA, trichloroacetic acid; Tricine, N-tris-(hydroxymethyl)-methylglycine.

INTRODUCTION

In the course of a study aiming at determining which proteins of the two chloroplast envelope membranes are coded by the plastid genome, we have noticed that no systematic study had been carried out to optimize the *in organello* proteosynthesis conditions for spinach chloroplasts. Such a study is particularly needed since, following the incorporation of radioactive precursors into intact chloroplasts, the labelling of envelope proteins is very low.

The purpose of this investigation is to improve the conditions under which proteosynthesis occurs in spinach intact chloroplasts. The optimal conditions which have been found differ greatly from those described for pea chloroplasts (*i.e.* Nivison and Jagendorf, 1984).

MATERIALS AND METHODS

Isolation of purified, intact chloroplasts. Intact chloroplasts were obtained as described by Siegenthaler

and Dumont (1990) from spinach (*Spinacia oleracea* L. var. Nobel) grown in phytotron (Siegenthaler and Depéry, 1976). To reduce the starch content, the leaves were left overnight in the cold room. The chloroplast suspension usualy contained 86 to 95% intact chloroplasts as determined by the rates of ferricyanide photoreduction in intact and osmotically lysed plastids (Heber and Santarius, 1970) and phase contrast microscopy. The chlorophyll concentration, obtained as indicated by Bruinsma (1961), was adjusted to 1 mg ml^{-1} with the assay medium.

Incubation conditions. We first tested the incubation media proposed by Ramirez *et al.* (1968) and Bottomley *et al.* (1974), which contained either 200 mM KCl, 66 mM Tricine-KOH (pH 8.3) and 6.6 mM $MgCl_2$ ("KCl" medium) or 330 mM sorbitol and 50 mM Tricine-KOH (pH 8.4) ("Sorbitol" medium). Various parameters were studied and the assay mixture was adapted according to the results obtained. The media were autoclaved before use and the glassware washed in chromic acid to minimize bacterial contamination. The chloroplast suspension (0.2-1.0 ml), containing chlorophyll at 1 mg ml^{-1}, was incubated with L-[^{35}S]methionine (1.85-5.55 MBq mg^{-1} Chl., specific activity >37 TBq $nmol^{-1}$, SJ-204, Amersham). The assay was carried out at 20°C in the water bath of a Gilson respirometer in the presence of a broad spectrum of red light (2.2 mW cm^{-2}). To avoid sedimentation, the test tubes were gently shaken. The light was turned off after 30 to 90 min and the tubes quickly transferred to ice. The chloroplasts were diluted fivefold with ice-cold assay medium and rapidly centrifuged at 4,480×*g* for 60 s (Sorvall SS-34 rotor). The supernatant was discarded and the thylakoid, stroma, inner and outer envelope membrane fractions isolated as described by Siegenthaler and Dumont (1990). The thylakoids were washed twice with S1 (10 mM Tricine-NaOH (pH 7.6), 5 mM $MgCl_2$, 2 mM EDTA-Na_2) by centrifugation at 7,840×*g* for 15 min in a SS-34 rotor, then resuspended in S1 containing 30% glycerol. After protein (Bradford, 1976 or Markwell *et al.*, 1981) and chlorophyll content determination, every fraction was stored in liquid nitrogen.

Measurement of radioactivity. Duplicate aliquots (5 µl) were taken out of the reaction mixture (containing intact chloroplasts) and out of the four chloroplast fractions. They were spotted onto filter papers and washed according to Ellis and Hartley (1982). Each filter was deposited into a scintillation vial containing 0.4% PPO, 0.01% POPOP in 2 ml toluene (w/v). The radioactivity of the TCA-insoluble material was measured in a Nuclear Chicago Isocap/300 liquid scintillation counter for 10 min.

Expression of results. The incorporation of [^{35}S]methionine in intact chloroplasts is expressed as cpm μg^{-1} chlorophyll. The amount of precursor incorporated depended on the ratio of [^{35}S]methionine to chlorophyll, on the specific activity, on the volume of the reaction mixture and also on the stepwise improvements of the method. Therefore, the results presented here are from typical experiments chosen among at least three assays. Experimental conditions are given in the legend of each figure.

RESULTS AND DISCUSSION

Optimization of the *in organello* incubation medium

In order to determine the optimal incubation conditions for proteosynthesis in intact spinach chloroplasts, we first tested the two basic media described by Ramirez *et al.* (1968) and Bottomley *et al.* (1974). We found that, during the first 20 min, the precursor uptake was linear in both media. However, because the "KCl" medium led to more [^{35}S]methionine uptake (data not shown), this medium was used thereafter to study the most important parameters (temperature, pH, concentrations of chlorophyll, cold amino acids, ATP, $MgCl_2$ and KCl) influencing the rate and extent of proteosynthesis.

Keeping the ratio [^{35}S]methionine to chlorophyll constant, several chlorophyll concentrations were tested. The total amount of ^{35}S incorporated increased with chlorophyll concentration up to 0.8-1 mg chl ml^{-1} then diminished due to chloroplast self-shading. The best incubation temperature was found to be 20°C; an increase of about 20% in total uptake was obtained when the assay medium was buffered at pH 7.6 whereas the incorporation peak in pea chloroplasts occurs at pH between 8.2 to 8.5 (Siddell and Ellis, 1975; Fish and Jagendorf, 1982). We also found that a 50 µM mixture of 19 L-amino acids, prepared as indicated by Reisfeld and Edelman (1982), stimulated the incorporation of the precursor by about 40-50%; a plateau was then reached for concentrations between 50 and 200 µM and, as also shown by Nivison and Jagendorf (1984), amino acid concentrations higher than 200 µM led to a steady decrease without, however, becoming inhibitory. The rate and time-course of the labelled amino acid incorporation in pea chloroplasts are higher when ATP-$MgCl_2$ is added (Nivison and Jagendorf, 1984); contrarily, the incorporation in spinach intact chloroplasts was

not stimulated by exogenous ATP but was rather slightly inhibited. The precise conditions specified by Nivison and Jagendorf (1984) were also found inhibitory (data not shown).

The relative effects of proteosynthesis parameters are differently modulated depending on the osmoticum chosen. This is well illustrated here by the $MgCl_2$ requirements which differred markedly between "KCl" and "Sorbitol" chloroplasts. As can be seen in figure 1 A, 2 mM $MgCl_2$ were required for optimal proteosynthesis in the presence of the "KCl" medium while all the concentrations tested were inhibitory when the "Sorbitol" one was used. Although very low Mg^{2+} concentrations (50-300 μM) stimulate proteosynthesis in pea chloroplasts resuspended in sorbitol (Nivison and Jagendorf, 1984), the results obtained suggest that spinach chloroplasts may have different requirements.

Figure 1 B shows that the rather low and hypotonic KCl concentration of 100 mM (114 mM after pH adjustment) greatly stimulated protein synthesis. Furthermore, the time-course of the incorporation was also substantially extended; even 50 mM KCl (64 mM final) led to a low but steadily increasing rate up to 90 min. Surprisingly, with concentrations of 200 and 300 mM KCl (214 and 314 mM final), net incorporation stopped after 30 and 20 min respectively.

Finally, the time-course of the incorporation of [^{35}S]methionine under our improved conditions was compared with the two previous basic "KCl" and

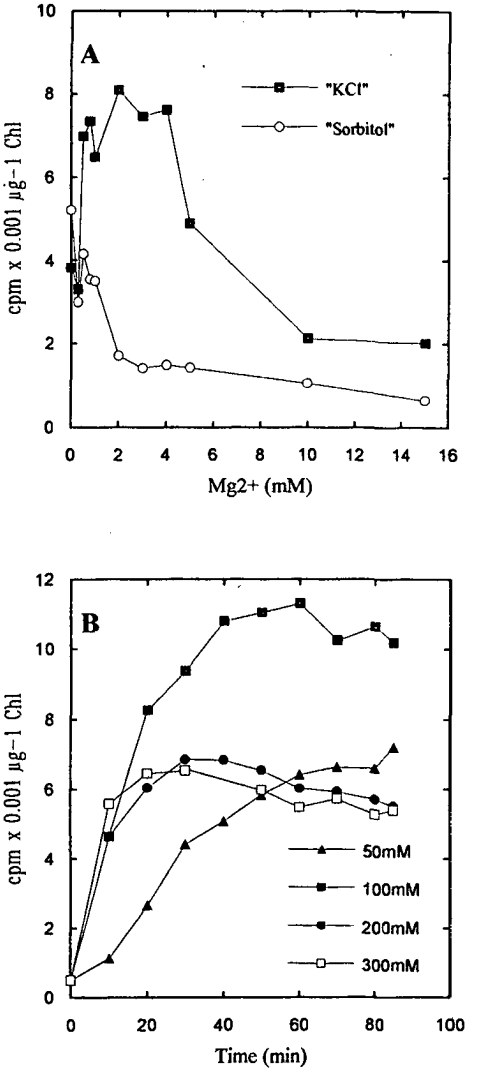

Figure 1. *Effect of $MgCl_2$* (**A**) *and KCl* (**B**) *on light-induced incorporation of [^{35}S]methionine into intact spinach chloroplasts.* **A**, the "KCl" and "Sorbitol" media (see Materials and Methods) containd 1 mg Chl ml^{-1}, 2.20 MBq [^{35}S]Met mg^{-1} Chl and various amount of $MgCl_2$ in a final volume of 500 μl; **B**, the assay medium (600 μl) contained 1 mg Chl ml^{-1}, 66 mM Tricine-KOH (pH 7.6), 2 mM $MgCl_2$, 50 μM cold amino acids, 2.4 MBq [^{35}S]Met mg^{-1} Chl and either 50, 100, 200 or 300 mM KCl. The results are expressed in cpm μg^{-1} Chl after 30 min (**A**) or as a function of time (**B**).

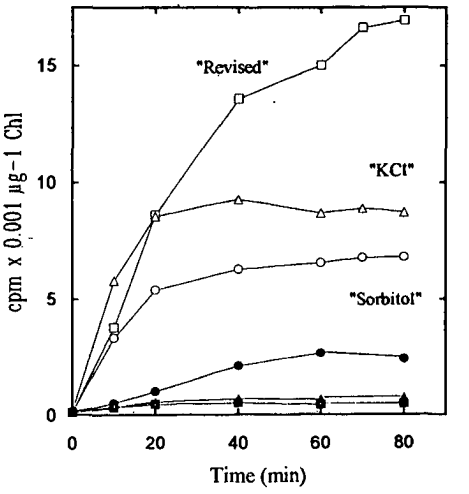

Figure 2. *Comparison of the incorporation of [^{35}S]methionine into intact spinach chloroplasts resuspended in "KCl" medium, "Sorbitol" medium and in the revised medium.* The revised medium contained 66 mM Tricine-KOH (pH 7.6), 100 mM KCl, 2 mM $MgCl_2$ and 50 μM cold amino acids. The "KCl" and "Sorbitol" media are as described in Materials and Methods. Each incubation medium contained 178 μM CHX, 1 mg Chl and 2.40 MBq mg^{-1} Chl in a final volume of 1 ml. (□, ■), Revised; (△, ▲), KCl; (○, ●), Sorbitol. Open symbols: light; closed symbols: dark. The incorporation is expressed in cpm μg^{-1} Chl as a function of time.

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Methods

Involvement of 70S ribosomes in the synthesis of some polypeptides constitutive of inner and outer envelope membranes of spinach chloroplast

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Abstract Proteosynthesis was performed *in organello*, using intact chloroplasts isolated from spinach (*Spinacia oleracea* L.) leaves to determine the extent of the plastid genome and 70S ribosomes involvement in the synthesis of chloroplast envelope proteins. The chloroplasts were ruptured in a hypertonic medium and the four fractions (thylakoids, stroma, inner and outer envelope membranes) purified. The proteins associated with each fraction were separated by temperature-induced phase partition in the presence of Triton X-114 according to their hydrophobic or hydrophilic characteristic. The products of protein synthesis were analysed by one and two dimensional electrophoresis as well as by autoradiography and immunology. Approximately 31 (9 major) and 36 (9 major) radioactive bands were detected in the inner and outer envelope membranes, respectively. There were 16 (9 major) bands in the thylakoid membranes and 42 (6 major) in the stroma. Five envelope polypeptides (66, 64, 45, 25 and 23 kDa) appeared to be of chloroplast origin. The 45 kDa protein was the most strongly labelled. The RubisCO large subunit was detected in the autoradiograms of the two membrane aqueous phases. Five other radioactive bands, shared in both the aqueous and organic phases, were shown to correspond to newly synthesized thylakoid polypeptides. The presence of those polypeptides is discussed in relation to their maturation.

Key words *In organello* proteosynthesis, 70S ribosomes, chloroplast envelope membranes, thylakoid, phase partition, *Spinacia oleracea*

Abbreviations CF1, coupling factor 1; 1-D, 2-D, electrophoresis in one or two dimensions; FBPase, fructose bisphosphatase; hsp70, 70 kDa heat shock protein; IEF, isoelectric focusing; IM, envelope inner membrane; LHCPII, light harvesting chlorophyll protein; LS, RubisCO large subunit; OM, envelope outer membrane; pI, isoelectric point; R 5-P kinase, ribulose 5-phospho kinase; RubisCO, ribulose 1,5-bisphosphate carboxylase/oxygenase; S, stroma; SS, RubisCO small subunit; T, thylakoid; TX-114, Triton X-114.

INTRODUCTION

The chloroplast envelope is the all important semi-permeable barrier between the cytosol and the stroma. Among the proteins present in the envelope are receptors, carriers, translocators as well as other enzymes implicated in lipid synthesis, etc. While the genomic origin of the stroma and the thylakoid membrane proteins is well-documented, there is very little corresponding information concerning the envelope membrane proteins. These represent 1 to 2% of the total chloroplast protein material (Douce and Joyard, 1979). The few envelope proteins which have been sequenced so far are of nuclear origin (Flügge *et al.*, 1989; Schnell *et al.*, 1990; Block *et al.*, 1991; Dreses-Werringloer *et al.*, 1991; Li *et al.*, 1991). However, the results of five studies which deal with proteins synthesized on chloroplast 70S ribosomes and include an analysis of the newly synthesized polypeptides of the whole envelope

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indicate that two to five polypeptides associated with the envelope are synthesized on chloroplast ribosomes (Joy and Ellis, 1975; Morgenthaler and Mendiola-Morgenthaler, 1976; Vasconcelos, 1976; Guéra *et al.*, 1989; Clemetson *et al.*, 1992).

The question of the genomic origin of the chloroplast envelope membrane proteins was reexamined using a spinach-specific incubation medium for *in organello* proteosynthesis (Dumont-BéBoux and Siegenthaler, 1993). Intact spinach chloroplasts were isolated and incubated in the presence of L-[^{35}S]methionine. The thylakoids, the stroma and the inner and outer envelope membranes were purified and their respective proteins were separated by SDS-PAGE and 2-D IEF–SDS-PAGE. Although the emphasis was put on the two envelope membrane proteins, a comparative study of the thylakoid and stroma radioactive products was also included as a control. Cross-contamination of radioactive proteins is a problem for a variety of reasons. Pure inner and outer envelope membranes (thylakoid-free) are difficult to obtain. Moreover, it is not yet possible to eliminate stroma components. As the two membranes cannot be separated at the level of the contact sites (Cline *et al.*, 1985), outer membrane proteins may account for up to 15% of the protein in an inner membrane sample (Block *et al.*, 1983 *a*; Werner-Washburn *et al.*, 1983; Keegstra *et al.*, 1984).

Previous work showed that Triton X-114 temperature-induced phase separation method is a useful tool to discriminate between the different types of membrane- encountered proteins (Siegenthaler and Dumont, 1990). The fractionation of a given set of proteins, prior to electrophoretic separation, leads to the formation of two phases enriched in either hydrophobic (organic phase) or hydrophilic (aqueous phase) proteins. This selective enrichment, which was verified by immunological studies, showed that the organic phases of the two envelope membranes were devoid of any stroma contaminants such as the RubisCO (Siegenthaler and Dumont, 1990), FBPase and R5-P kinase (Dumont-BéBoux, 1991). In contrast, those enzymes were detectable in the aqueous phases and the controls.

In this investigation, the envelope-associated radioactive products obtained after proteosynthesis *in organello* were studied and compared with those from stroma or thylakoids by combining TX-114 preliminary separation and electrophoresis methods.

RESULTS

SDS-PAGE

Figure 1 shows the Coomassie blue profiles (A) and the corresponding autoradiogram patterns (B) obtained with proteins processed by temperature-induced phase separation and SDS-polyacrylamide gel electrophoresis. As illustrated in figure 1 A, the proteins of the four fractions partitioned according to their hydrophobic or hydrophilic characteristic and were found enriched in either the organic (detergent-rich) (*fig.* 1 A: T/LHCP, 32,20; IM/34) or the aqueous (detergent-poor) phase (*fig.* 1 A: T/30; OM/109,25). However, the autoradiogram results (*fig.* 1 B) suggest that the newly synthesized proteins, which were detected in the two phases of the membrane fractions (*fig.* 1 B: T/32,29,20; IM,OM/60,45) may not partition according to their relative hydrophobicity. It should be noted that, in contrast to the organic phases of the membranes, the organic phase of the stroma normally contained two labelled bands only, one at 60 kDa and the other at 54 kDa. The ambiguous partition of the newly labelled proteins was most evident in fractions from either the inner or outer envelope membranes. Radioactive polypeptide bands showed similar electrophoretic mobility but opposite behaviour with respect to the Triton X-114 partitioning (*e.g.* strong labeling in the IM organic phase and the OM aqueous phase).

In order to assess the validity of the phase partition method for newly synthesized proteins, the radioactive profiles of the four fractions were analysed. They were also compared with one another to verify any comigration of polypeptides.

Thylakoid protein analysis

The autoradiogram in figure 1 B showed that the control (*fig.* 1 B: T/c) contained 9 major labelled bands (indicated by their molecular mass) and about 7 minor ones. These 16 bands were then distributed between the two phases (*fig.* 1 B: T/o,a; *fig.* 2 A and B). As could be expected, thylakoid hydrophobic polypeptides like the 32 kDa (D1/cyt f) and the 29 kDa (D2?), illustrated in figures 1 B (T/o) and 2 B (T/o; pl 5.65-6.7), were enriched in the organic phase. Likewise, the two hydrophilic CF1 subunits α and β (60 and 54 kDa), identified by immunology (data not shown) and 2-D electrophoresis, were detected almost exclusively in the aqueous phase (*fig.* 1 A and B; *fig.* 2 A and B: T/a, pl 6.44 and 6.57).

The strong radioactive band at 15 kDa displayed the same electrophoretic mobility as the CF1 ϵ subunit. However, it presented a strong hydrophobic character whereas immunological work showed that the CF1 ϵ subunit had a hydrophilic character and partitioned mostly in the aqueous phase (data not presented).

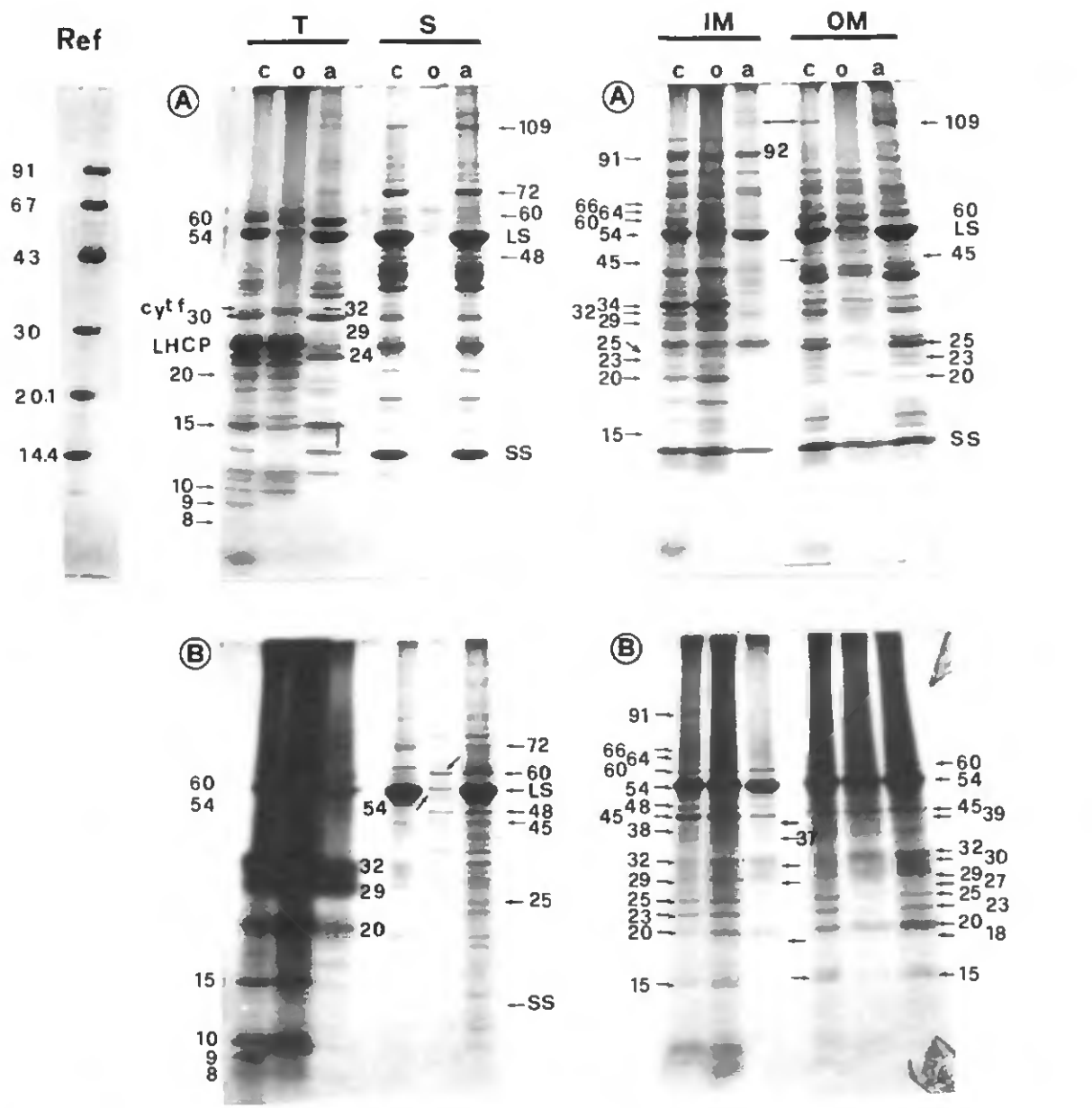


Figure 1. *Electrophoretic* (A) *and radioactive* (B) *patterns of thylakoid membranes* (T), *stroma* (S), *inner* (IM) *and outer* (OM) *envelope membrane proteins after proteosynthesis* in organello *and TX-114 temperature-induced phase separation.* c, Triton X-114-untreated control; o, organic phase; a, aqueous phase. Ref, reference proteins. Molecular masses are indicated in kDa. The separating SDS-gel contained a 10 to 18% acrylamide gradient. The controls contained 50 µg proteins and 100 µg proteins were used for the Triton X-114 phase separation. In **B**, the Kodak X Omat AR film was exposed for 4 weeks at room temperature.

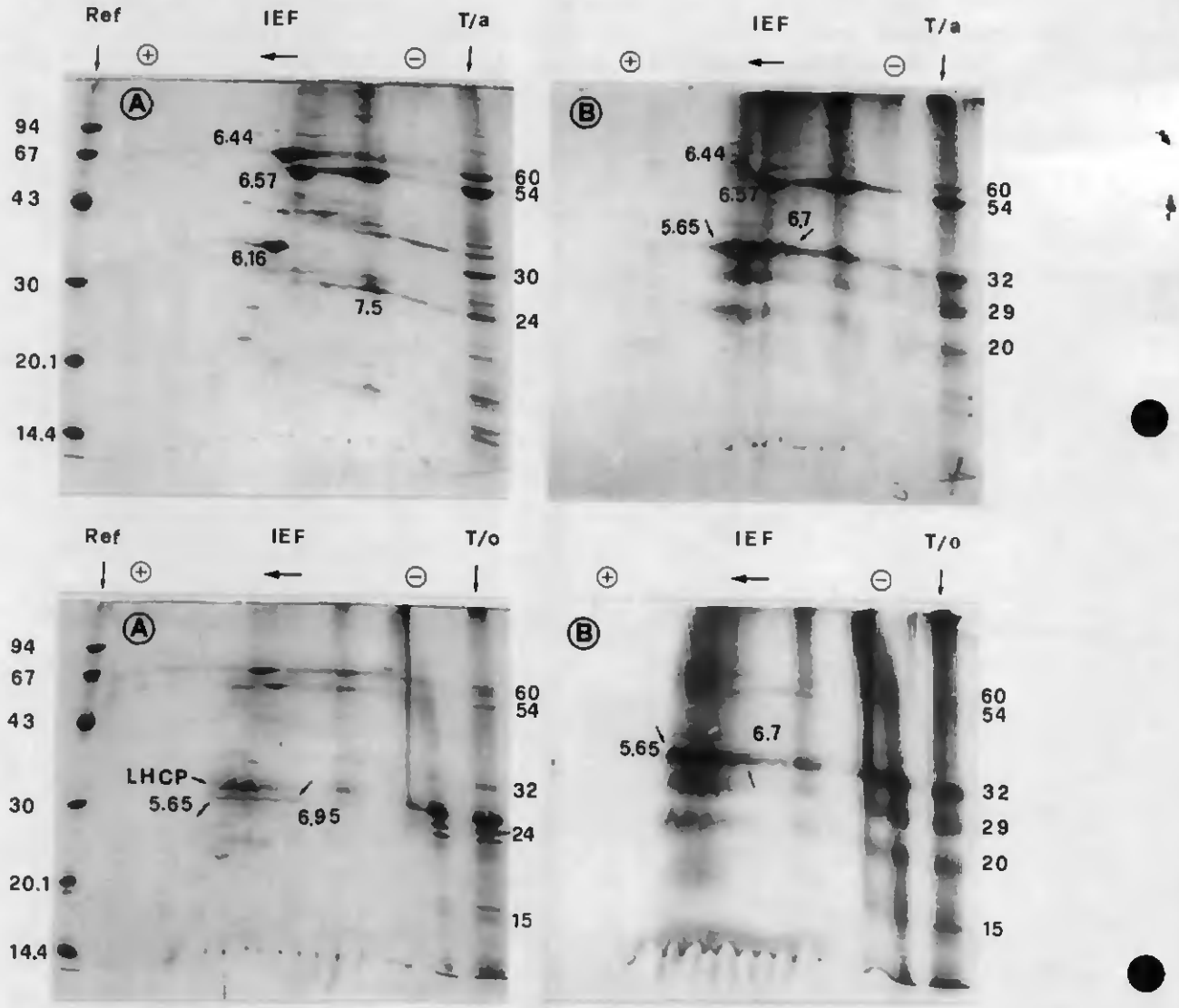


Figure 2. *Two-dimension electrophoretic separations of the aqueous and organic phases of the thylakoid proteins.* Coomassie blue stained gels (**A**) and autoradiograms (**B**). T/a and T/o, aqueous and organic controls in 1-D. Ref, reference proteins. Molecular masses are indicated in kDa and numbers on the figures refer to pI. The separating SDS-gels of the vertical second dimension contained 13% polyacrylamide. The thylakoid phase partition was obtained from 150 µg proteins ; in **B**, Kodak X Omat AR films were exposed for 4 weeks at room temperature.

Stroma protein analysis

The stroma is composed of many soluble proteins and enzymes. However, transiting polypeptides of nuclear origin, plastid translation products of diverse site fate, and proteins belonging to the envelope intermembrane space may also be found in the stroma. Figure 1 B (S/c,o,a) shows that the essential of the [^{35}S]label was recovered in the aqueous phase, as expected. The absence of label in the RubisCO small subunit confirmed that our preparations did not contain any active 80S ribosomes. Two labelled polypeptides, at 60 and 54 kDa, were repeatedly detected in the organic phase (arrows in *fig.* 1 B: S/o).

Another distinguishing feature is the strong label found at 72 kDa in the aqueous phase. By 2-D electrophoretic separation (*fig.* 3 A), this polypeptide band was resolved into two spots (pI 5.8 and 6.5), both immunologically unrelated (*fig.* 3 C and D). Furthermore, as only one was labelled, they differred

in their genomic origin too (*fig.* 3 B). The unlabelled one (pI 5.8) was also detected in the stroma of pea (*fig.* 3 E) and was shown to be immunologically homologous to a pea hsp70 (Bovet *et al.*, 1990) (data not presented).

Inner and outer envelope protein analysis

Both the phase partition and the labelling of the newly synthesized proteins associated with the two envelope membranes are illustrated in figure 1 A and B. The radioactive profiles (*fig.* 1 B: IM, OM/c,o,a) were similar in spite of the fact that the Coomassie blue electrophoretic patterns of the TX-114-untreated samples were different (*fig.* 1 A: IM,OM/c). Furthermore, the proteins did partition according to their hydrophobic or hydrophilic characteristic (*fig.* 1 A: IM,OM/o,a). This is particularly well illustrated by the nuclear-encoded 34 kDa polypeptide of the inner membrane (*fig.* 1 A: IM/o,a) which partitioned exclusively in the organic phase. As the radioactive patterns of the two controls (*fig.* 1 B: IM,OM/c) were very much alike, a similar partition of the radioactive polypeptides between the aqueous and organic phases of the two membranes was to be expected. However, most of the radioactivity bound to the inner membrane was found in the organic phase (*fig.* 1 B: IM/o) whereas the outer membrane labelling was mostly recovered in the aqueous phase (*fig.* 1 B: OM/a). Furthermore, Coomassie blue counterparts were either weak or absent. The radioactive results presented in figure 1 B as well as others obtained with different SDS-PAGE systems (data not shown) also indicated that the labelled polypeptides at 60, 54, 32, 29, 20 and 15 kDa had similar electrophoretic mobility as polypeptides found in the stroma (60, 54 kDa) and/or the thylakoids. Figure 1 B shows that a very high number of radioactive bands were associated with the envelope membranes. The major ones presented molecular masses of 60, 54, 48, 45, 32, 29, 25, 23 and 20 kDa and the minor ones 91, 66, 64, 39, 38, 37, 30, 27, 18 and 15 kDa.

IEF – SDS-PAGE

To further characterize the labelled proteins and identify potential contaminants, 2-D electrophoresis was performed on fractions either treated or not treated with Triton X-114. The autoradiograms obtained with the organic and the aqueous phases of the two envelope membranes were very similar to one another (data not shown), thus indicating that the difference in the label distribution between the phases (more label in the IM organic phase and in the OM aqueous phase) could not be explained by the presence of unrelated proteins presenting similar molecular

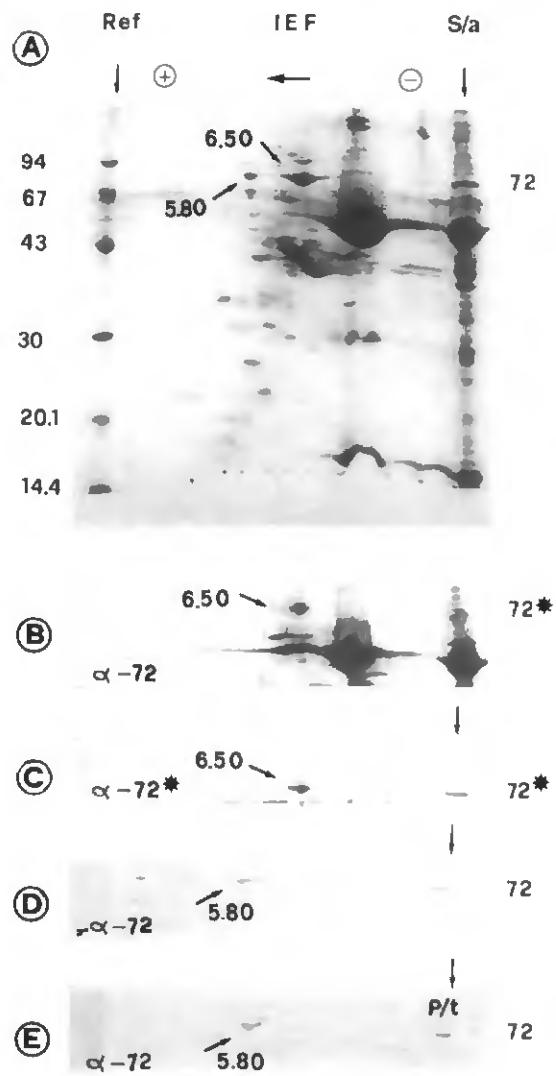
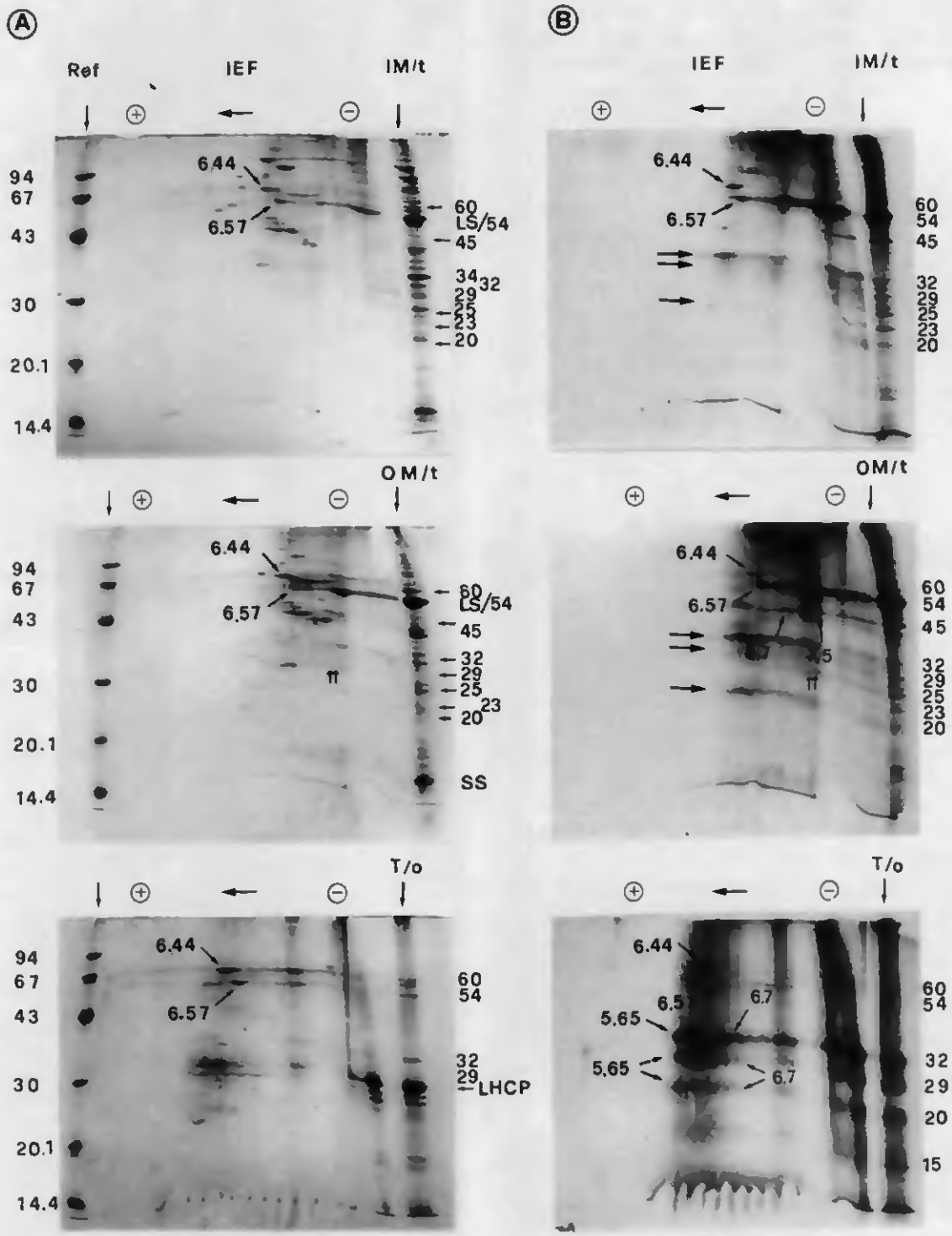


Figure 3. *Two-dimension electrophoretic separations of the aqueous phase of the stroma proteins.* Coomassie blue stained (**A**), autoradiogram (**B**) and immunoblots in the presence of antibodies raised against the chloroplast-encoded 72* kDa (**C**, α-72*) and the nuclear 72 kDa of spinach stroma (**D, E**, α-72). S/a, spinach aqueous control in 1-D; P/t, total pea stroma in 1-D. Ref, reference proteins. Molecular masses are indicated in kDa and numbers on figures refer to pI. In **B, C, D** and **E**, only the relevant parts are shown. The separating SDS-gels of the vertical second dimension contained 13% acrylamide. In **B**, the autoradiogram was obtained after 6 weeks of exposure.


A
Ref
IEF
IM/t
94
67
43
30
20.1
14.4
6.44
6.57
60
LS/54
45
34
32
29
25
23
20
OM/t
SS
T/o
LHCP
B
5.65
6.7
15

masses. A comparison with the autoradiogram of the stroma aqueous phase showed that three radioactive spots were in common, namely the RubisCO large subunit, the 60 and the 54 kDa polypeptides (data not shown). Moreover, the autoradiograms of the organic and aqueous phases of the membranes also revealed the same radioactive pattern as their two untreated controls which are presented in figure 4, along with the autoradiogram of the thylakoid organic phase. Figure 4 shows that five strong radioactive traces, found at 60 kDa (pl 6.44), 54 kDa (pl 6.57), 32 (pl 5.65-6.7), 29 and 20 kDa (pl 5.65-6.7), were detected in all three IEF – SDS-PAGE separations. The 60 and 54 kDa traces alone had fairly well-defined counterparts in the Coomassie blue profiles of the inner and outer envelope membranes (*fig.* 4 A: IM,OM). Along with the RubisCO large subunit, these two polypeptides were also detected on the 2-D electrophoretic separation of the stroma aqueous fraction. The three lower molecular mass traces (thick arrows on *fig.* 4 B: IM,OM) presented pl ranges slightly shorter than those found in the thylakoid membranes at 32, 29 and 20 kDa but had otherwise similar profiles.

Among the many other radioactive bands discovered on the 1-D electrophoretic separations of the two membranes, only the 45 and the 25 kDa polypeptides were still detectable in the 2-D separations. Both of them were absent from the thylakoid fraction (see also *fig.* 2 B) and only weakly present in the stroma (*fig.* 1 B: S/c,a). In the outer membrane IEF – SDS-PAGE separation, the 45 kDa polypeptide was resolved as a long trace with three possible spots, one at the origin, one at pl 7.5 and one at about 6.7 (*fig.* 4 B: OM). None of these spots had any clear counterparts on the Coomassie profiles (*fig.* 4 A: IM,OM). Most of the radioactive material stayed at the origin in the Triton X-114-untreated IM fraction (*fig.* 4 B: IM), but migrated as in the outer membrane in the IM organic and aqueous phases (data not shown). Finally, the 25 kDa polypeptide displayed a pl of about 7.5 and was mainly detected in the outer membrane autoradiogram (double arrow in *fig.* 4 B: OM). It is also noteworthy that a weakly stained counterpart was visible on the corresponding Coomassie blue stained gel (double arrow in *fig.* 4 A: OM).

DISCUSSION

The *in organello* approach to study the fate of polypeptides synthesized on 70S ribosomes gives an overall view of the precursor distribution between the four chloroplast fractions. The adaptation of an incubation medium which is spinach-specific (Dumont-BéBoux and Siegenthaler, 1993) has caused a substantial increase of the [^{35}S]methionine incorporation, leading to the detection of several not-yet-recorded radioactive polypeptidic bands in the two envelope membranes. In this study, the already well-characterized thylakoid and stroma proteins have been successfully used as a control to verify the degree of reliability of the temperature-induced phase separation method on newly synthesized proteins as well as the presence of radioactive proteins unique to the envelope.

The analysis of the thylakoid fraction has shown that the Triton X-114 temperature-induced phase partition method is a valid tool to separate newly synthesized membranous and peripheral proteins into pools enriched in either hydrophobic or hydrophilic polypeptides. Yet, because of the high level of radioactive detection, the partition does not always appear as clear-cut as with the bulk of "cold" proteins. When quantitatively important proteins (*e.g.* CF1 subunits) or proteins with a high turnover (*e.g.* D1) are considered, radioactive traces may "overflow" in the opposite phase. This is in agreement with results of Bricker and Sherman (1982). However, the newly synthesized proteins associated with the two envelope membranes have a more equivocal partition, differing from the "overflowing" effect detected in the thylakoids. Depending on whether they are associated with the inner or the outer membrane, these otherwise identical proteins display opposite

Figure 4. *Two-dimension electrophoretic separations of the inner and outer envelope and thylakoid membrane proteins.* **A**, Coomassie blue stained gels; **B**, corresponding autoradiograms. The inner and outer envelope membranes were not treated with Triton X-114. IM/t, OM/t, 1-D SDS-PAGE of inner and outer envelope membrane proteins; T/o, 1-D SDS-PAGE of the organic phase of the thylakoid membrane proteins. Ref, reference proteins. Molecular masses are indicated in kDa and numbers on figures refer to pl. The separating SDS-gels of the vertical second dimension contained 13% acrylamide. The thylakoid phase partition was obtained from 150 µg proteins ; 100 µg of total inner and outer membrane proteins were used for the IEF – SDS-PAGE. In **B**, the Kodak X Omat AR films were exposed 8 weeks for IM and OM autoradiograms and 6 weeks for the thylakoid one.

behaviour in Triton X-114 (*fig.* 1 B: IM,OM/o,a). This is particularly well illustrated with the 15 kDa radioactive trace which appears in the organic phase of both the thylakoid and the inner membrane but only in the aqueous phase of the outer membrane. This could be linked to the outer membrane lipid/protein ratio which, at 3, is very high compared to 0.8 in the inner membrane and 0.4 in the thylakoid membranes (Block *et al.*, 1983 *b*) and to the ratio of Triton to lipid during phase partition. More investigation is needed to understand the role that the membrane lipid/protein ratio might play in such a temperature-induced phase partition of proteins. However, it means that the protein partition obtained with the inner membrane is likely to give a better representation of the hydrophobic/hydrophilic character of the proteins than that obtained with the outer membrane.

The IEF – SDS-PAGE separations have confirmed that some newly synthesized thylakoid proteins are found associated with the two envelope membranes. This result was unexpected as immunological studies showed that the two membranes were free from any LHCPII (data not presented) and that, if and when chlorphyll was detectable, it was only in trace amounts (Dumont-BéBoux, 1991). The presence of these thylakoid proteins is particularly surprising in the outer membrane as this fraction never contained any chlorophyll. Among the five polypeptides concerned, the first two (60 and 54 kDa) are stained by Coomassie blue, strongly labelled, and have molecular mass and pl similar to those of the α and β CF1 subunits. They are detected also in the thylakoid-free stroma fraction. The other three polypeptides (32, 29, 20 kDa) have similar electrophoretic characteristics as D1, D2 (T29) and T20. Like D1 and D2, the two polypeptides migrating at 32 and 29 kDa are not visible on Coomassie blue stained gels. Confirmation of the similarity of these proteins was also obtained by the use of a Bio Image analyser. The composite images obtained on the Bio Image screen by comparing the 2-D autoradiograms of the thylakoid with those of the inner and outer envelope membranes indicate that these proteins (60, 54, 32, 29 and 20 kDa) do indeed match. As suggested by the absence of immunological response towards the LHCPII antibodies and by the absence of chlorophyll, the association of thylakoid polypeptides with the envelope membranes involves only newly synthesized proteins; their association with the envelope might be the reflection of a physiological stage related to their maturation process. It would be transient only while interacting with molecular chaperones such as the hsp-related 72 kDa (also detected in the inner membrane) or the chaperonin implicated in the maturation of proteins and/or the mechanism of nuclear and plastidial subunit association. This association of foreign labelled proteins with the envelope is in contradiction with the results described for pea (Joy and Ellis, 1975), *Euglena gracilis* (Vasconcelos, 1976) and *Chlamydomonas reinhardtii* chloroplasts (Clemetson *et al.*, 1992). However, since the first two studies were made, the characterization methods have been greatly improved.

The 45 kDa polypeptide (*fig.* 4 B: IM,OM), on the other hand, is absent from any of the thylakoid phases and is only weakly detected in the stroma. Since it is very strongly detected in the two envelope membranes, it is a very good candidate for an envelope polypeptide encoded by the plastid genome and synthesized on 70S ribosomes. It will be difficult to further characterize this protein as a hydrophobic or hydrophilic polypeptide because it was detected in both phases of the two membranes. However, if one considers the appropriate partition to be that of the inner membrane newly synthesized proteins, then a hydrophobic character can be assigned to this protein (*fig.* 1 B: IM/o). Furthermore, as no counterpart can be detected in the organic phases of the inner and outer membranes (*fig.* 1 A: IM,OM/o), it is either a very minor component with many methionine residues or one presenting a large turnover. The Triton X-114 phase separation results suggest that at least two different polypeptides migrate at 45 kDa: a hydrophobic one, not visible on Coomassie blue stained gels, corresponding to our labelled 45 kDa and a hydrophilic one, detectable by Coomassie blue and not labelled. This is supported by the discrepancy between the respective intensities of the staining and the labelling at 45 kDa in the organic and aqueous phases of the inner membrane (*fig.* 1 A and B: IM/o,a).

Of the radioactive components which were weakly or undetectable by 2-D electrophoresis, the polypeptides at 66, 64, 25 and 23 kDa are solely detected in the envelope membranes and can also be considered as potential candidates for envelope polypeptides encoded by the plastid genome. The latter two radioactive bands (25 and 23 kDa), which have matching Coomassie blue bands in the outer membrane, might belong to this structure.

The above study of the genomic origin of the chloroplast envelope proteins shows the following

main features: (*a*) several envelope polypeptides are labelled during *in organello* proteosynthesis, among which 9 major and at least 10 minor ones; (*b*) out of the 9 major polypeptides, 5 are found in other compartments of the chloroplast; (*c*) polypeptides having similar molecular mass and pl are distributed in detergent-free or -enriched phase (*e.g.* the labelled polypeptides of OM partition mainly in the aqueous phase whereas these of IM are recovered in the organic phase); (*d*) by a combination of several experimental approaches, it can be concluded that of all the plastid-encoded polypeptides, 5 are detected in the envelope only.

The presence of plastid-encoded proteins in the envelope has now been demonstrated in a variety of organisms, from green alga to higher plants. Although most of the genetic information has been transferred to the nucleus, it is obvious that some has been retained and that each chloroplast compartment possesses proteins which are synthesized on 70S ribosomes.

Taken together, these results point out the diverse and dynamic nature of the chloroplast membrane biogenesis as well as the constant interaction between different plastid compartments.

METHODS

Isolation of purified, intact chloroplasts. Intact chloroplasts were obtained as described by Siegenthaler and Dumont (1990) from 800 g of spinach leaves (*Spinacia oleracea* L.) bought at the local market. The chloroplasts, stored on ice, were resuspended in the incubation medium at a chlorophyll concentration of 2 mg ml^{-1}. The suspension contained between 86% and 95% intact chloroplasts as determined by the rates of ferricyanide photoreduction in intact and osmotically lysed plastids (Heber and Santarius, 1970) and phase contrast microscopy.

Proteosynthesis *in organello*. L-[^{35}S]methionine (specific activity > 37 TBq $nmol^{-1}$, SJ-1515, provided by Amersham) was incorporated in intact chloroplasts equivalent to 30 to 45 mg chlorophyll. Just before use, the chloroplast suspension was ajusted to 1 mg ml^{-1} with the incubation medium which contained 166 mM KCl, 66 mM Tricine-KOH (pH 7.6) and 2 mM $MgCl_2$. The incorporation was performed essentially as described by Dumont-BéBoux and Siegenthaler (1993). The suspension was preincubated 15 min in the light and under gentle shaking in a large flat-bottomed Erlenmeyer in the presence of 178 μM cycloheximide. The cold mixture of 19 amino acids (50 μM each) and the precursor (185 MBq [^{35}S]Met) were then added in the dark and the chloroplast suspension was distributed among sterile plastic vials at the rate of 1 ml. The incubation (60 min) was performed in a water bath at 20°C in subdued white light (40 W m^{-2}) provided by 6 bulbs (100 W each) placed 25 cm below the bottom of the vials. The lamps were cooled with a fan. Those conditions gave results similar to those obtained with red light. At the end of the incubation time, the light was turned off and the tubes immediately transferred to ice. The chloroplasts were diluted fivefold with ice-cold incubation medium and treated according to published protocols (Dumont-BéBoux and Siegenthaler, 1993). The four chloroplast fractions (thylakoid, stroma, inner and outer envelope membranes) were isolated as described by Siegenthaler and Dumont (1990).

Antibody preparation. Two antibodies against stroma polypeptides (α-72 and α-72*) were raised in our laboratory by making three intradermic inoculations to white New-Zealand rabbits at intervals of 2 to 3 weeks. The antibodies against the 72 polypeptide (α-72) were obtained by isolating the desired polypeptide bands by SDS-PAGE of the stroma control fraction. The gel bands were pooled and crushed in liquid nitrogen before being dissolved in the complete Freund's adjuvant, diluted 1 : 1 with distilled water. Two boosts were made with the same amount of antigene emulsified in the incomplete Freund's adjuvant. The α-72* was obtained from the corresponding antigene separated by 2-D electrophoresis (IEF – SDS-PAGE). After transfer of the proteins onto nitrocellulose paper by the semi-dry technique, the antigene was prepared for inoculation as indicated by Knudsen (1985).

Other techniques. Protein temperature-induced phase separation in the presence of Triton X-114 was performed as indicated by Siegenthaler and Dumont (1990); isoelectric focusing and SDS-PAGE, molecular mass determination, Western blotting, autoradiography and immunological studies have also been described (Siegenthaler and Dumont, 1990). The protein content was measured according to Bradford (1976) or Markwell *et al.* (1981) and the chlorophyll determined as indicated by Bruinsma (1961). The gels were stained as described by Neuhoff *et al.* (1988).

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EXPERIENTIA 43, 660, 1987

Which polypeptides of inner and outer chloroplast envelope membranes are coded by chloroplast DNA?

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It is well established that chloroplast proteins are coded either by the chloroplast and/or the nuclear genome. Here, we investigate optimal conditions ($MgCl_2$, KCl, pH, temperature, time, etc.) under which protein synthesis takes place in intact spinach chloroplast in the presence of ^{35}S-methionine as protein precursor. After isolation of pure inner and outer envelope membranes, polypeptides of each fraction are separated by SDS-gel electrophoresis. Using fluorography and sectioning techniques, labeled polypeptides are tentatively identified in both membrane fractions arising from control and chloramphenicol-treated intact chloroplasts. The degree of radioactivity incorporation and the pattern of labeled polypeptides are presented for both inner and outer membrane fractions. Results will be discussed in terms of the chloroplast DNA contribution in building up envelope proteins compared to those of thylakoid membranes.

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SEPARATION OF STROMAL AND ENVELOPE MEMBRANE PROTEINS OF SPINACH CHLOROPLASTS INTO HYDROPHILIC AND LIPOPHILIC FRACTIONS

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The purpose of this investigation is to characterize and differentiate between the proteins of the chloroplast stroma and inner (IM) and outer (OM) envelope membranes which are encoded by the chloroplast genome. To this aim, protein synthesis was carried out in intact chloroplasts under controlled conditions in the presence of ^{35}S-methionine as protein precursor. Stroma and membrane proteins were separated according to their hydrophobic or hydrophylic (or more loosely bound) properties by Triton X-114 phase partition. Proteins of the IM fraction are mostly found in the detergent phase while the OM proteins are essentially present in the aqueous phase. Only a few stromal proteins in the range of 45 to 65 kD are recovered in the organic fraction. Among the proteins coded by c-DNA, the 75 and 45 kD stromal proteins, the 36 kD protein of the IM and the 33 and 16 kD proteins of the OM are strongly labeled. 2D-electrophoresis and immunological techniques have been used to characterize further the localization of these proteins.

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SEPARATION OF STROMAL, THYLAKOID, INNER AND OUTER ENVELOPE MEMBRANE PROTEINS OF SPINACH CHLOROPLASTS INTO HYDROPHILIC AND HYDROPHOBIC FRACTIONS

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INTRODUCTION

The chloroplast contains about 400 structural or functional polypeptides, out of which up to 20 to 30% could be coded for and synthesized in the plastid (1). They are distributed between the stroma, the thylakoid, and the envelope membrane (lumen, inner and outer membranes) and give, when separated on SDS-PAGE, characteristic but very complex profiles. Due to this complexity, two (or more) otherwise different polypeptides may comigrate and thus contribute to a single Coomassie band. We have used Triton X-114 (TX-114) phase partition, essentially as described by Bordier (2), so as to obtain a preliminary separation of the polypeptides according to their hydrophobic/hydrophilic properties. Additional techniques such as proteosynthesis in organello in the presence of ^{35}S-methionine (3), 2D-electrophoresis (4) and immunology (5) were used to go further into the identification of specific proteins that could have been otherwise mistaken for other comigrating ones.

RESULTS AND DISCUSSION

After isolation of intact spinach chloroplasts, four fractions were obtained mostly according to (6): thylakoid, inner and outer envelope membranes and stroma. The polypetides belonging to the envelope lumen should now be found in the stroma and/or the inner and outer envelope membranes, depending on the interactions involved. These four fractions were submitted to TX-114 phase partition and then separated by SDS-PAGE (fig.1). As expected, all but four of the stroma polypeptides partitioned in the aqueous phase and the recovery was excellent. The recovery was also quite good in the membranes when strongly hydrophobic or hydrophilic polypeptides were concerned: the thylakoid 32 and 23 kDa, the inner membrane 34 kDa were exclusively recovered in the organic phases, while the outer membrane 109, 40, 16 and 14 kDa partitioned in the aqueous phase. Therefore, by discriminating between hydrophobic and hydrophilic polypeptides, TX-114 enhances the SDS-PAGE resolution. However, Coomassie bands often have a counterpart with identical Mr in the other phase, in which case, we may be dealing either with two or more different polypeptides or with peripheral ones

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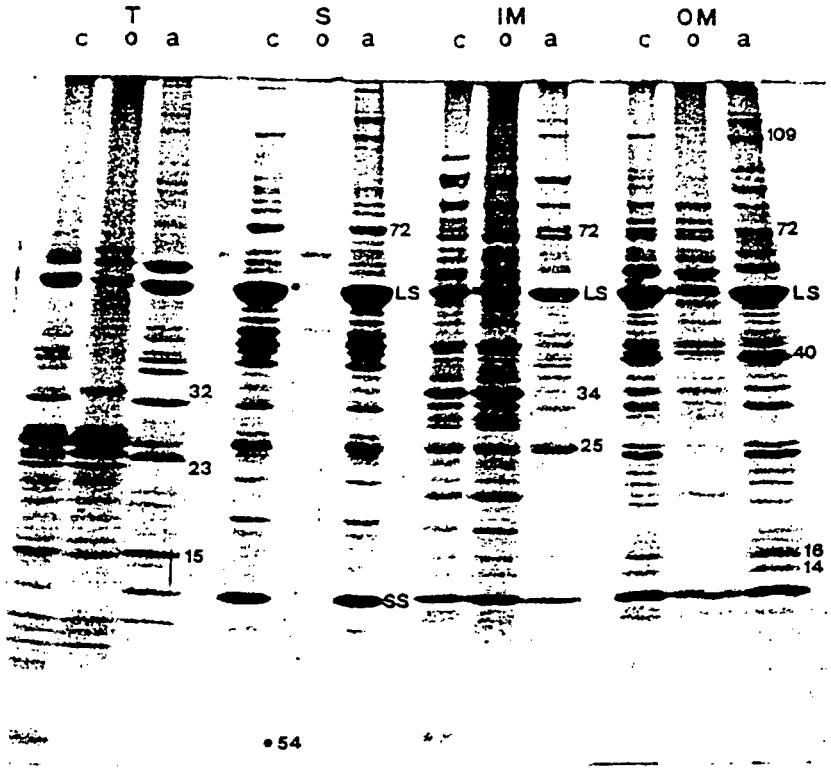
that are not totally separated from the membrane (ie: T15; IM72, 25; OM72). Furthermore, a unique hydrophobic or hydrophilic Coomassie band may still contain more than one polypeptide. Three cases of comigration (T15, S72, S,IM,OM 54) will be discussed below.

Fig. 1 SDS-PAGE of the four fractions after TX-114 phase partition



T: thylakoid S: stroma IM: inner membrane OM: outer membrane
c: untreated control (30 g) o: organic phase a: aqueous phase
LS: RubisCO large subunit SS: RubisCO small subunit
100 g of each fraction were submitted to TX-114 phase partition
(one third of the stroma aqueous phase was loaded)

Case 1: Thylakoid 15 kDa
As seen in Fig.1, the 15 kDa Coomassie band partitioned unequally in the two phases, the aqueous band being much more important than the corresponding one in the organic phase. To determine whether there was more than one polypeptide, the separation was repeated after proteosynthesis. The autoradiograph indicated that the hydrophobic polypeptide was labelled while the hydrophilic comigrating one was not. Therefore, the Coomassie band which, before TX-114 treatment, appeared

as unique was in fact composed of two polypeptides of different genomic origin and different hydrophobic/hydrophilic properties.

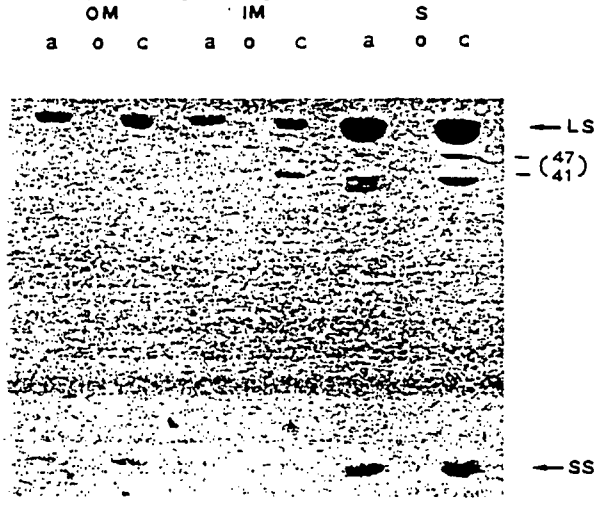
Case 2: Stroma 72 kDa

Fig.1 shows that the stroma 72 kDa was clearly hydrophilic. On 2D-electrophoresis, it was resolved into two spots with pI ~6.5 and ~6.3 respectively, which might be charge isomeres of a unique polypeptide. We have raised a polyclonal antibody against the aqueous 72 kDa polypeptide and tested it on 2D-electrophoresis. Only the polypeptide with pI ~6.3 gave a positive reaction. 2D-electrophoresis was then repeated with ^{35}S-methionine-labelled material. The autoradiograph showed that the polypeptide with pI ~6.5 (which was not immuno-reactive) was labelled. Therefore, the single Coomassie aqueous band in 1D-electrophoresis (7) was composed of two different comigrating polypeptides with different genomic origin but identical behaviour toward TX-114 phase partition.

Case 3: Stroma, Inner and Outer envelope membranes 54 kDa

After proteosynthesis and TX-114 phase partition, a radioactive 54 kDa band was found in the organic phases of the stroma, the inner and outer envelope membranes at the level of the RubisCO large subunit (LS) (fig.1). The RubisCO is a known contaminant of the envelope membrane and has been, so far, impossible to eliminate. As the TX-114 partitioning was shown to be reliable, we tried to find out whether this 54 kDa Coomassie band was the LS or not. First, an antibody against the RubisCO (kindly provided by Dr. A. Radunz) was tested on the three treated fractions as well as on their respective controls (fig.2).

Fig. 2: Immunoblot of the three fractions after TX-114 treatment



Symbols as in Fig.1 (only the relevant part of the immunoblot is shown)

Fig.2 shows that a positive reaction was found only in the controls and the aqueous phases of the three fractions. No reaction was seen on any organic phase. Furthermore, a polyclonal antibody was raised in our laboratory against the polypeptide found in the stromal organic phase. When tested, it did not react with LS in any of the aqueous phases but reacted with all three organic phases. There is therefore a fair probability that the hydrophobic polypeptide, uncovered in the three fractions by TX-114 treatment, was not the large subunit. If so, the RubisCO, known to resist sonication and other drastic treatments (8), could be eliminated from the envelope membranes by TX-114 phase partition.

CONCLUSIONS

Owing to the ability of Triton X-114 to bind to integral hydrophobic polypeptides by replacing most of the lipid molecules and together with temperature-induced phase partition, it is possible to separate membraneous polypeptides having close electrophoretic mobility but different behaviour towards TX-114 phase partition as well as possible stromal contaminants. Indeed, as the separation is excellent when one deals with either very hydrophobic or very hydrophilic polypeptides, any soluble stromal contaminant will be recovered in the aqueous phase. Furthermore, the organic phase polypeptides are exclusively of membrane origin while those from the aqueous phase may have a double origin, namely stroma and membrane. However, comigration may still exist in one of the two phases and furthermore, in some cases, the phase partition is not clear cut. The latter may be due either to unfavorable hydrophilic/hydrophobic balance or to incomplete solubilization and may therefore be indicative of peripheral or extrinsic polypeptides. It is then necessary to use other criteria, such as those illustrated here, to differentiate between polypeptides with similar electrophoretic mobility but with identical or ambivalent behaviour towards TX-114 phase partition. Taken together, these methods represent a valuable preparative step towards the isolation, purification and characterization of membraneous spinach chloroplast polypeptides (9).

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POSSIBLE PRESENCE OF A 75 kDa HEAT SHOCK POLYPEPTIDE IN THE STROMA OF SPINACH CHLOROPLAST

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High temperature stress or heat shock induces dramatic changes in gene expression in a wide variety of prokaryotic and eukaryotic organisms. In plants (soybean seedling tissues) several heat shock proteins (hsp) characterized by a low (less than 30 kDa) or higher (65 to 85 kDa) Mr have been identified (Key et al., PNAS US 78, 3826-30, 1981). Recently, Vierling et al., PNAS US 83, 361-5, 1986) have demonstrated that in soybean, pea and corn, nuclear-coded hsp having a Mr of 21 and 27 kDa are transported into chloroplasts. We shall report on a 75 kDa polypeptide encountered in the spinach chloroplast stroma. The antibodies raised against this polypeptide crossreact with the stroma fraction of three different plant species (tobacco, lettuce, pea) as well as with one of the three hsp 70 forms found in bovine muscle. On the other hand, antibodies against a highly conserved region of mammalian hsp 70 s react with a pea stromal polypeptide equivalent to the 75 kDa spinach polypeptide. Some of the biochemical and immunological properties of the spinach 75 kDa polypeptide will be presented. Supported by NSF 31.26386.89.

Résumé

Il est bien établi que les deux génomes, nucléaire et chloroplastique, coopèrent pour le codage des protéines plastidiales. Le génome chloroplastique du Tabac ayant été élucidé, on sait qu'il contient, entre autres, l'information de plusieurs protéines du thylacoïde et du stroma. Il existe cependant encore des "open reading frames" (ORF) dont la fonction n'est pas connue et qui sont susceptibles de coder des protéines membranaires. Peu d'études se sont portées sur l'origine génomique des protéines de l'enveloppe. Les quelques-unes qui ont été caractérisées sont d'origine nucléaire mais il est cependant possible que d'autres soient d'origine plastidiale et correspondent à des ORF. C'est ce que nous avons cherché à vérifier dans ce travail. Pour ce faire, nous avons commencé par optimaliser le milieu d'incubation utilisé pour la protéosynthèse *in organello* (chapitre 1). Les résultats de l'incorporation en présence de ^{35}S-méthionine, présentés dans le chapitre 2, montrent que, après séparation électrophorétique en une dimension, des bandes polypeptidiques radioactives, détectables par fluorographie et comptage direct, apparaissent dans les fractions des membranes interne et externe de l'enveloppe. Pour parvenir à une première caractérisation de ces protéines radioactives, nous avons utilisé une méthode de partition de phases en présence de Triton X-114 qui permet de séparer les protéines associées aux membranes en deux "sets", composés respectivement de protéines intrinsèques (phase organique) et de protéines périphériques et solubles (phase aqueuse). Cette méthode, mise au point dans le chapitre 3, a été ensuite appliquée à du matériel radioactif (chapitre 4). D'autres critères de séparation, de détection et de caractérisation (électrophorèses en deux dimensions, autoradiographies et immunologie) ont également été utilisés. Après analyse des résultats, il s'est avéré que, parmi les protéines associées aux membranes de l'enveloppe et paraissant avoir été synthétisées sur les ribosomes 70S, quelque huit sont susceptibles de leur appartenir. En cours de travail, nous avons

été amenée à nous intéresser à deux protéines du stroma et le chapitre 5 est consacré à cette étude. Une de ces protéines stromatiques et trois des huit protéines citées plus haut ont été isolées en vue d'un séquençage. Les méthodes de purification et les résultats des analyses d'acides aminés sont présentés dans le chapitre 6.

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