

Triton X-114 phase-fractionation of maize thylakoid membranes in the investigation of thylakoid protein topology

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1. INTRODUCTION

Integral membrane proteins can be separated from peripheral proteins by temperature-precipitated phase-fractionation in Triton X-114 solutions [1]. Proteins are separated by their ability to form mixed micelles with Triton X-114; only proteins with extensive hydrophobic regions (i.e., integral polypeptides) can form mixed micelles with non-ionic detergents [2,3].

We have utilized this procedure to assist in the investigation of the topological orientation of maize chloroplast thylakoid membrane proteins by following enzymatic radioiodination or the limited tryptic digestion of intact maize thylakoid membranes with phase-partitioning. Proteins which partition into the Triton X-114 phase and are susceptible to tryptic degradation or radioiodination, are considered to be integral proteins possessing surface-exposed domains. Our results indicate that all of the major, identified integral polypeptides partition into the Triton-phase and have surface-exposed sites accessible to enzymatic radioiodination and/or tryptic degradation. The major proteins which partition into the aqueous phase include the α -, β - and γ -subunits of CF₁, and polypeptides of 32, 25 and $14 \times 10^3 M_r$.

2. MATERIALS AND METHODS

Chloroplast thylakoid membranes were isolated as in [4] from 9–12-day-old maize seedlings and either used immediately or after storage at 80°C. Trypsin treatment was performed on intact maize

thylakoid membranes essentially as in [5], treatment was at 37°C for 15 min at 2, 10 and 40 g/ml. The reaction was stopped by adding 20-fold excess soybean trypsin inhibitor and 1 mM PMSF.

Lactoperoxidase-mediated radioiodination was performed on intact membranes at 2 mg membrane protein/ml in 10 mM Tricine–NaOH (pH 7.5) containing protease inhibitors (1 mM PMSF, 1 mM caproic acid, 1 mM benzamidine). The iodination mixture contained (in 1 ml): 5 mM MgCl₂, 10 mM D-glucose, 7.2 mU glucose oxidase/ml, 373 mU lactoperoxidase/ml and 100 Ci ¹²⁵I NaI. Radioiodination was allowed to proceed for 1 h under room light at 21°C, and the reaction was stopped by the addition of excess buffer containing 1 mM sodium thiosulfate. The labeled membranes were harvested by centrifugation and washed twice in 10 mM Tricine–NaOH (pH 7.5). Under the gentle iodination conditions described above the reaction was highly specific for externally localized protein domains [20]. The structural intactness of the membrane was tested by treating the membrane with Triton X-100 prior to iodination. In all cases this treatment resulted in the iodination of many more proteins (see [12]). The omission of lactoperoxidase resulted in an incorporation of ¹²⁵I that was <1% of the incorporation in its presence. Autoradiography was performed on Kodak X-OMAT AR film at –80°C.

After either enzymatic radioiodination or trypsin treatment, phase partitioning with Triton X-114 was performed as in [1]. Thylakoid membranes were suspended at 2 mg protein/ml in a solution containing 1% pre-washed Triton X-114,

150 mM NaCl, 1 mM EDTA and 10 mM Tris-HCl (pH 7.6) for 30 min. The solubilized membranes were centrifuged at $12000 \times g$ for 1 min to remove a starch pellet and then phase partitioned at 37°C for 5 min. The cloudy mixture was centrifuged for 3 min at $12000 \times g$ and yielded a heavy, detergent-enriched fraction (Triton phase) and a light, detergent-depleted fraction (aqueous phase). The fractions were separated and the Triton phase was washed with the above buffer (minus Triton X-114) while the aqueous phase was brought to 1% Triton X-114. Phase partitioning was repeated on both fractions at least twice.

Lithium dodecyl sulfate-PAGE was performed as in [6] in 10–20% gradient gels at 0°C , and proteins were detected after staining with Coomassie blue. Cytochromes were detected by heme-dependent peroxidase activity [6]. Protein was determined by the Lowry method [7] as modified [8].

3. RESULTS

Fig. 1 illustrates the result of a typical phase-fractionation of maize thylakoid membranes. The pattern of fractionation of the majority of the polypeptides is highly complementary; polypeptides either partition into the aqueous phase or the Triton phase but not both. Of the major identified polypeptides, the subunits of CF_1 (α, β, γ) and a M_r 32000 polypeptide partition strongly into the aqueous phase while the apoproteins of CP I and LHCP, the PSII reaction center polypeptides I (M_r 49000) and II (M_r 45000), and cytochromes f and b_6 partition into the Triton phase. The majority of the remaining proteins partition into the Triton phase, although a few polypeptides partition into both phases. This apparent failure to partition completely may be due to co-migration of polypeptides which differ in their phase separation characteristics. Alternatively, polypeptides having extensive hydrophilic regions and relatively small hydrophobic regions may partition in this manner. It also appears that certain, predominantly aqueous partitioning polypeptides, such as the α and β subunits of CF_1 spill over into the Triton phase. This may be due to the large amount of these polypeptides present. It is also possible that a small amount of CF_1 remains associated with CF_0 and is carried into the Triton phase.

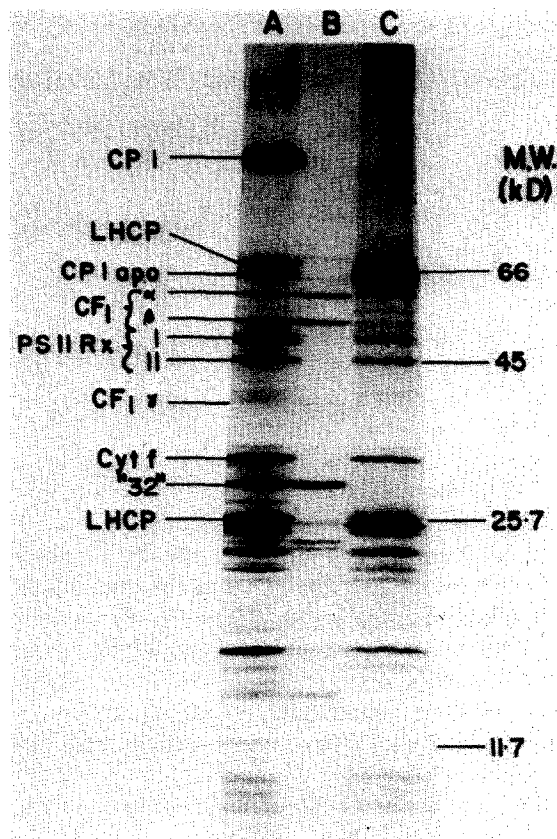
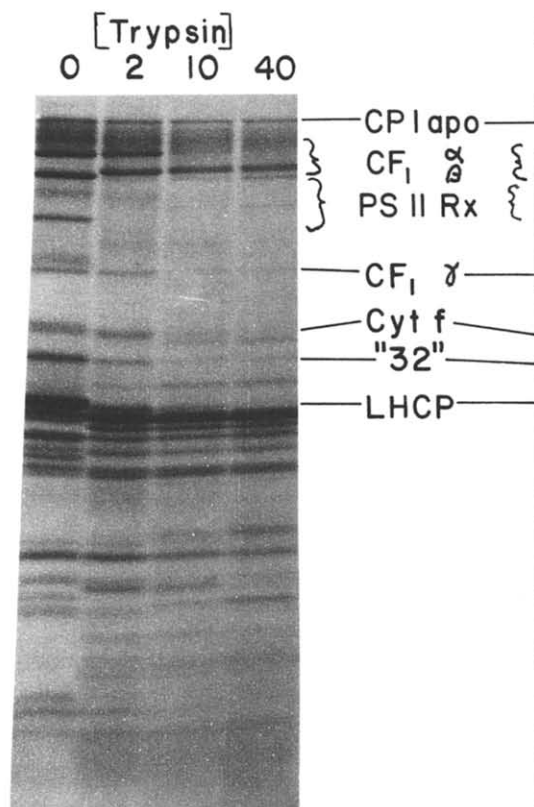


Fig. 1. Typical Triton X-114 mediated partitioning of maize thylakoid membranes: (A) membranes; (B) aqueous phase; (C) Triton X-114 phase.

All of the polypeptides which partition into the aqueous phase are eventually affected by trypsin treatment (fig. 2). Concomitantly, there is an accumulation of presumptive tryptic fragments in the $19\text{--}24 \times 10^3 M_r$ region and at lower- M_r -values in the aqueous phase. A number of polypeptides which partition into the Triton phase are also affected by trypsin treatment. These include the PSII reaction center polypeptides I and II, with the lower- M_r component (II) being digested at lower trypsin concentrations than the higher- M_r polypeptide I. The main LHC apoprotein exhibits its characteristic loss of $M_r \sim 2000$ as reported by others [5]. Under these treatment conditions, both cytochrome f and b_6 lose $M_r \sim 1000$ (fig. 2, insert) during treatment with higher trypsin concentrations. There is also an accumulation of apparent tryptic fragments in the lower- M_r regions of the

A. MEMBRANES



B. PHASE FRACTIONATION

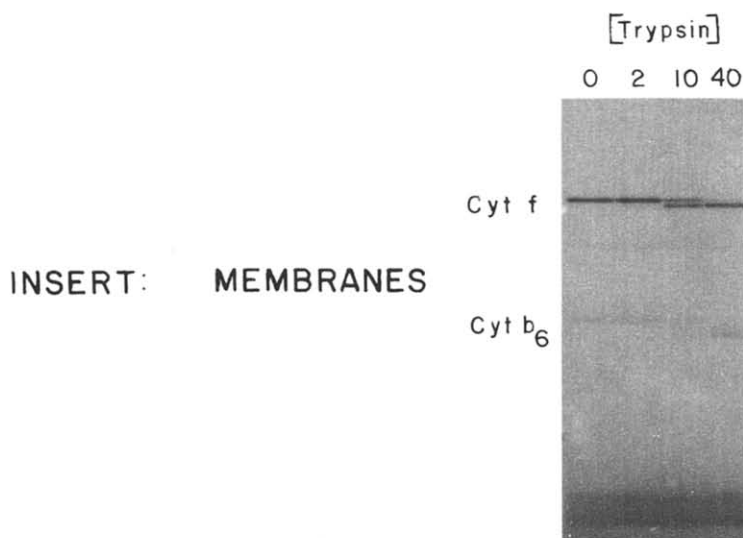
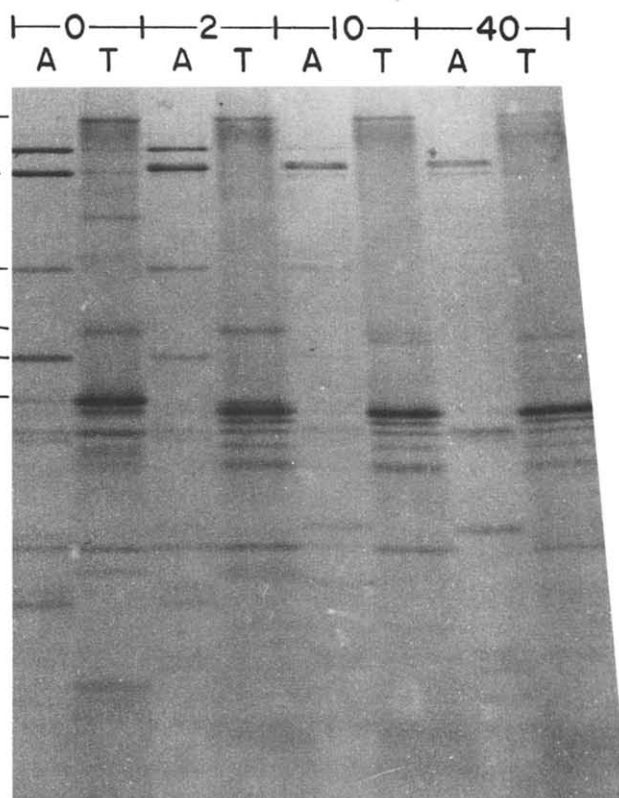


Fig. 2. Trypsin treatment of maize thylakoid membranes: (A) membranes treated with stated trypsin concentrations; (B) trypsin-treated membranes followed by phase fractionation. (A) aqueous phase; (T) Triton X-114 phase. Insert: Membranes treated with various trypsin concentrations and stained for cytochromes by heme-dependent peroxidase activity.

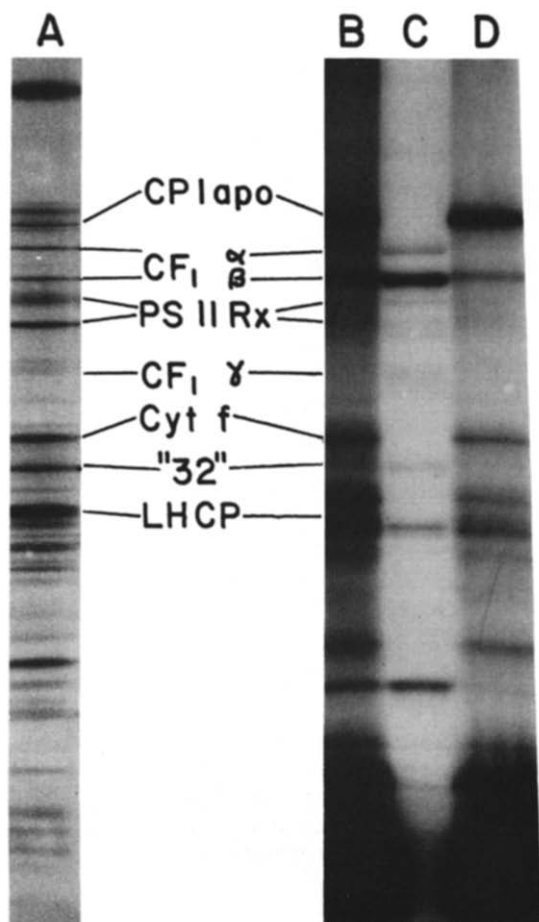


Fig. 3. Lactoperoxidase-catalyzed iodination of maize thylakoid polypeptides: (A) profile of membrane proteins after Coomassie bleu staining; (B) membrane proteins radioiodinated with lactoperoxidase; aqueous-phase (C) and Triton X-114 phase (D) polypeptides after lactoperoxidase-catalyzed iodination.

Triton X-114 fractions, particularly at $M_r \leq 10000$.

The enzymatic radioiodination of intact thylakoid membranes is illustrated in fig. 3. The principal polypeptides labeled in the aqueous phase include the α and β subunits of CF_1 , and the M_r 32000 polypeptide. The β subunit is much more heavily labeled than the α subunit. Other labeled polypeptides are found in the $40\text{--}50 \times 10^3 M_r$ region, at M_r 25000 and at $M_r \sim 14000$. The major radiolabeled Triton-phase polypeptides include the CP1 apoprotein and cytochrome f . A small but detectable amount of label also appears to be associated with the lower- M_r PSII reaction center

polypeptide II. Numerous other polypeptides are labeled in the region at $M_r < 30000$. Four polypeptides are labeled in the vicinity of the major LHC apoprotein ($20\text{--}26 \times 10^3 M_r$), although the major LHC apoprotein does not appear to be labeled. The results of phase fractionation, trypsin treatment and enzymatic radioiodination are summarized in table 1.

4. DISCUSSION

Triton X-114-mediated phase fractionation allows the unambiguous assignment of the majority of membrane polypeptides to either an integral (Triton phase) or peripheral (aqueous phase) membrane domain [1]. We have utilized this procedure to assist in elucidating the topological arrangement of the major thylakoid membrane proteins in maize. Membranes were first labeled with ^{125}I or treated with trypsin and then phase fractionated with Triton X-114. The major intrinsic polypeptides resolvable in our system are amphiphilic molecules possessing surface-exposed sites which can be enzymatically radioiodinated and/or tryptically degraded. These polypeptides include the CP1 apoprotein, the PSII reaction center polypeptides I and II, cytochromes f and b_6 , and the major LHC apoprotein.

The exposure of the PSII reaction center polypeptides I and II to tryptic attack and, in the case of polypeptide II, radioiodination suggests surface exposure of these polypeptides. This is consistent with the early observation [9] that low levels of radioiodination preferentially inhibited the PSII reaction center as measured by a quenching of the PSII variable fluorescence. Since we observe that only polypeptide II is radioiodinated, this may be the site of PSII inhibition in [8]. Polypeptide II is also more sensitive to trypsin treatment than is polypeptide I.

Our observation of the radioiodination of cytochrome f confirms and expands the report [10] of surface localization of this polypeptide, based on [^{35}S]DABS labeling. This result was not unexpected as both methods label exposed tyrosyl residues. The radioiodination of cytochrome f and the tryptic sensitivity of both cytochromes seems to suggest that the plastoquinol-plastocyanin oxidoreductase (cytochrome b_6/f particle [11]) is surface-exposed.

Table 1

Summary of the phase fractionation, radioiodination, and tryptic sensitivity of the identified polypeptide components of intact maize thylakoid membranes

Polypeptide	Phase-fractionation	Radio-iodination	Trypsin sensitivity
Apo CP 1	TX	+++	0
CF ₁ α	AQ	+	+++
CF ₁ β	AQ	+++	+++
PS II, '49 $\times 10^3 M_r$ '	TX	0	+
PS II, '45 $\times 10^3 M_r$ '	TX	+	++
CF ₁ γ	AQ	++	+++
Cytochrome <i>f</i>	TX	++	++, loss of M_r 1000
'32 $\times 10^3 M_r$ ' Coomassie blue-stainable	AQ	++	++
Major LHCP	TX	0	++, loss of M_r 2000
25, 24, 14 $\times 10^3 M_r$	AQ	++	+++
Cytochrome <i>b₆</i>	TX	n.d.	++, loss of M_r 1000

Polypeptides labeled TX-phase fractionate into the Triton X-114 phase, those labeled AQ fractionate into the aqueous phase. The key for radio-iodination or trypsin sensitivity is: +++, extensive; ++, moderate; +, low; 0, no modification under the conditions of section Z; n.d., iodination of this polypeptide has not been determined. The proteins are listed in order of decreasing apparent M_r .

The phase fractionation of thylakoid membranes generally degrades the chlorophyll-proteins to the level of their respective apoproteins. Thus, our finding that the CP1 apoprotein is heavily labeled during radioiodination differs from [12] where only small amounts of radioiodination were associated with this polypeptide in *Vicia faba* and *Hordeum vulgare*. This may be an example of a species-specific difference which can be substantial [12]. The major LHC apoprotein does not appear to be enzymatically radioiodinated. However, polypeptides of slightly lower and higher mobility are labeled, and these may be minor LHC apoproteins. In both *Chlamydomonas* and spinach, at least 5 polypeptides in the region appear to be associated with the LHC apoproteins [13]. A similar situation may exist in maize. The function of these additional polypeptides is unknown.

Among the peripheral (aqueous partitioning)

polypeptides, the β subunit of CF₁ is the major radioiodinated species. It exhibits much more extensive labeling than the α subunit [12]. Since the tyrosine content of both subunits is nearly identical [14], this differential labeling suggests that either the α subunits' tyrosines lie in the interior of the molecule or that they are shielded from radioiodination by the other CF₁ subunits (probably the β component).

Interestingly, a Coomassie blue-stainable M_r 32000 polypeptide, partitions into the aqueous phase. This polypeptide appears identical to the Coomassie blue-stainable polypeptide that is greatly reduced in the Mendelian mutant *hcf-3* [4,15] and to the M_r 32000 species isolated in [19]. This polypeptide is trypsin sensitive and moderately radioiodinated. These results are indicative of a surface-exposed extrinsic polypeptide. Polypeptides in this M_r region have been implicated in

atrazine binding [9], the organization of PSII [16] and possibly water oxidation [17–19]. The complete partitioning of this polypeptide into the aqueous phase may represent an excellent first step in the purification of this M_r 32000 polypeptide in its 'native' state, and may assist in the elucidation of its function.

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