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ISOELECTRIC POINTS OF SPINACH THYLAKOID MEMBRANE SURFACES AS DETERMINED BY CROSS PARTITION

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Summary

The isoelectric points of unbroken chloroplast lamellae and various subchloroplast fractions, including a preparation of inside-out thylakoids, have been determined using aqueous two-phase systems containing dextran and charged polyethylene glycol. When the amounts of material in the top phase in a phase system with the positively charged trimethylamino polyethylene glycol are plotted against pH the curve intersects the corresponding curve obtained from phase systems with the negatively charged polyethylene glycol sulfonate. This cross-point can be correlated with the isoelectric point of the material.

The cross-point for unbroken chloroplast lamellae was found to be around pH 4.7. Mechanical disintegration lowered the cross-point to around pH 4.4, probably because of exposure of new membrane surfaces. The disintegrated chloroplasts were fractionated by differential centrifugation to separate the grana and stroma lamellae. The stroma lamellae vesicles showed the same isoelectric point as the unbroken lamellae, while a cross-point at pH 4.3 was obtained for the grana-enriched fraction. For thylakoid membranes destacked under low salt conditions the cross-point was 0.3 pH unit lower than for membranes originating exclusively from the stroma lamellae. The most acidic cross-point (pH 4.1) was observed for the fraction enriched in inside-out grana thylakoids. It is suggested that the differences in isoelectric point between various subchloroplast fractions reflect a heterogeneous arrangement of surface charge along and across the thylakoid membrane.

Introduction

The surface charges of biological membranes are of great importance for protein-membrane and membrane-membrane interactions which are essential for

many biological structures and functions. For the chloroplast lamellae a lateral heterogeneity of charged groups has been suggested as playing an important role in its characteristic differentiation into regions with stacked membranes alternating with regions of separated membranes [1,2]. The present study is an attempt to investigate the distribution of charged groups both along and across the thylakoid membrane system. This has been done by isolating subchloroplast fragments representing different surface regions of the chloroplast lamellae, including the inner grana thylakoid surface, followed by determination of their isoelectric point by cross partition. By this method the isoelectric point is determined by partition in aqueous polymer two-phase systems having different interfacial potential [3,4]. Cross partition has previously been used for the determination of the isoelectric point of proteins [5,6] and mitochondrial membranes [7].

The results show that unbroken chloroplast lamellae (class II) have an isoelectric point at pH 4.7. Mechanical disintegration lowered the cross-point to around pH 4.4 probably because of exposure of new membrane surfaces. The disintegrated chloroplasts were fractionated by differential centrifugation to yield a separation of the grana and stroma lamellae. The stroma lamellae vesicles showed the same isoelectric point as the unbroken lamellae while a cross point at pH 4.3 was achieved for the grana-enriched fraction. For thylakoids membranes destacked under low salt conditions the cross-point was 0.3 pH unit lower than for membranes originating exclusively from the stroma lamellae. The most acidic cross-point (pH 4.1) was observed for the fraction enriched in inside-out grana thylakoids. These differences in isoelectric point between the various subchloroplast fractions demonstrate a heterogeneous arrangement of surface charge along and across the thylakoid membrane.

Materials

Polyethylene glycol was obtained from Union Carbide, New York, NY, U.S.A. and dextran 500, batch No. 5996 from Pharmacia Fine Chemicals AB, Uppsala, Sweden. Trimethylamino polyethylene glycol and polyethylene glycol sulfonate, synthesised from polyethylene glycol 4000 [8], was a gift from Dr. G. Johansson of this department.

Unbroken chloroplast lamellae were isolated from spinach as described earlier [9] and finally suspended in 50 mM sodium phosphate buffer (pH 7.4)/ 25 mM NaCl/0.3 M sucrose.

High salt thylakoid membrane fragments were obtained by passage of chloroplast lamellae, suspended in 50 mM sodium phosphate buffer (pH 7.4)/ 150 mM NaCl, twice through a Yeda press at a nitrogen gas pressure of 10 MPa. The Yeda press homogenate was fractionated by differential centrifugation consequetively at $1000 \times g$ for 10 min (1K), $10\,000 \times g$ for 30 min (10K), $40\,000 \times g$ for 30 min (40K) and $100\,000 \times g$ for 60 min (100K). The 100K fraction consisted of Photosystem I vesicles originating from the stroma lamellae, while the 10 K fraction mainly consisted of grana and was only slightly enriched in Photosystem II [9].

Low salt thylakoid membrane fragments were obtained by passage of chloroplast lamellae, suspended in 5 mM sodium phosphate buffer (pH 7.4)/2.5 mM

NaCl/100 mM sucrose, twice through the Yeda press at a nitrogen pressure of 10 MPa. After fractional centrifugation at 40 000 × *g* for 30 min and 100 000 × *g* for 60 min the lighter fraction (designated Ls100K) was used for cross partition.

Inside-out thylakoid membrane vesicles were isolated by phase partition as recently described [10,11]. This preparation method includes centrifugation of a high salt Yeda press homogenate at 40 000 × *g* for 30 min and suspension of this grana-enriched fraction in 10 mM sodium phosphate buffer (pH 7.4)/5 mM NaCl/100 mM sucrose followed by passage twice through the Yeda press. Finally, 5 ml of this suspension (800 μg chlorophyll/ml) was added to 20 g of a polymer mixture to yield a two-phase system of the following composition: 6.1% (w/w) dextran 500, 6.1% (w/w) polyethylene glycol 4000, 10 mmol/kg sodium phosphate buffer (pH 7.4), 5 mmol/kg NaCl and 20 mmol/kg sucrose. After the phase system had been mixed and settled the bottom phase (B) contained inside-out thylakoid vesicles while the top phase (T) contained mainly right-side-out vesicles as revealed by proton translocation [10] and freeze-fracture studies [11]. Besides these differences the B vesicles were highly enriched in Photosystem II properties [12] and showed a high density of large particles on their EF fracture faces indicating a granal origin [11]. The material was removed from the polymers by centrifugation for 3 h at 100 000 × *g* before the cross partition. All preparative work was performed at 4° C.

Methods

Two series of phase systems were prepared, one with positively charged polyethylene glycol and one with negatively charged polyethylene glycol. Final concentrations, in a 4 g system after addition of 0.2 ml sample, in series I were: 6.8% (w/w) dextran 500, 2.7% (w/w) trimethylamino polyethylene glycol 4000, 4.1% (w/w) polyethylene glycol 4000, 2.5 mM sodium phosphate buffer (initial pH 7.4), 1.25 mM NaCl, citric acid to yield desired pH (<5 mM), 100 mM sucrose (300 mM sucrose for unbroken chloroplast lamellae) and chloroplast material corresponding to 10 μg chlorophyll/ml.

Final concentrations in series II were the same as for series I except that polyethylene glycol sulfonate was used instead of trimethylamino polyethylene glycol.

We included the charged polymers in the phase system rather than different salts in order to achieve a larger portential difference between the phases [6,8]. With the charged polymers the partition in the two series will be drastically different even close to the isoelectric point of the membranes, which therefore can be determined more accurately than if only salts were included.

After addition of sample the phases were mixed and allowed to separate for 30 min. All partition work was performed at 4° C. From each phase 750 μl was withdrawn and diluted with an equal volume of water. The amount of material in the top phase was calculated from the absorbance at 680 nm of the diluted material. The pH was measured on the residual phase system after dilution with 2.5 ml water.

Results

Fig. 1 shows the result from cross-partition of the various thylakoid membrane populations, where the curves with closed circles represent series of phase systems with trimethylamino polyethylene glycol (positively charged) and the curves with open circles series of phase systems with polyethylene glycol sulfonate (negatively charged). The pH at which the two curves intersect is called the cross-point. At this pH the distribution of the material to each phase is independent of whether a negatively or positively charged polyethylene glycol is used. This means that the particles show no net charge to the surrounding polymers and therefore this pH is an estimate of the isoelectric point of the exposed membranes surfaces. Fig. 1 summarizes the cross-points

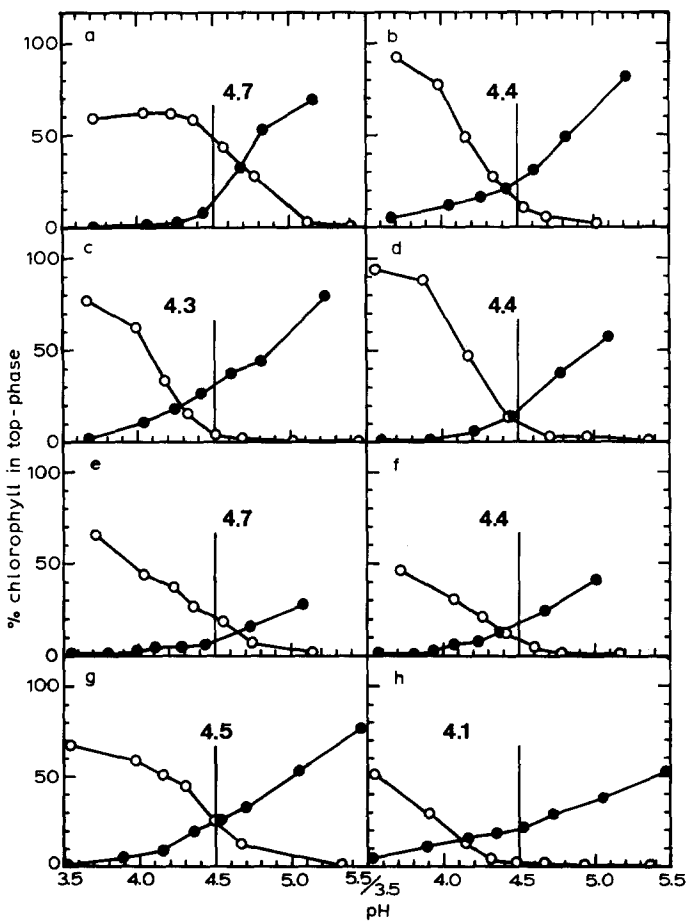

Fig. 1. Cross-partition of (a) intact chloroplast lamellae, (b) Yeda press homogenate, (c) 10K centrifugal fraction, (d) 40K centrifugal fraction, (e) 100K centrifugal fraction, (f) low salt fragments Ls100K, (g) T-material, and (h) B-material (inside-out). Closed circles represent the series of phase systems with trimethylamino polyethylene glycol (positively charged) and the open circles the series of phase systems with polyethylene glycol sulfonate (negatively charged). The corss-point values given are mean values from 2 to 4 determinations, and the deviation was not more than ±0.1 pH unit.

observed for the unbroken chloroplast lamellae and the various subchloroplast fractions.

For the unbroken chloroplast lamellae a cross-point at 4.7 was obtained. The Yeda press homogenate showed a cross-point that was 0.3 pH unit lower. Obviously new membrane surfaces with a lower isoelectric point were exposed by the disintegration procedure.

By differential centrifugation of the press homogenate a separation of the stroma lamellae and the stacked membranes of the grana was achieved [9]. From the cross-partition experiments on these centrifugal fraction the following results are of special interest: (a) The light centrifugal fraction (100K) containing membranes rich in Photosystem I originating from the stroma lamellae showed a cross-point at pH 4.7; a cross point at pH 4.3 was obtained for the grana-enriched heavy fraction 10K. (b) The cross-point of the stroma lamellae membranes (100K) was the same as for the unbroken chloroplast lamellae, confirming that this part of the thylakoid membrane, rich in Photosystem I, is exposed to the surrounding medium in the unbroken lamellae. (c) The low pH at the cross-point, obtained for the 10K fraction suggests that the new membrane surfaces exposed by the press treatment mainly originate from the grana region. Thus, it is reasonable to assume that the exposed membrane surfaces represent the partition region of the grana, which explains why they do not give any contribution to the cross-point of the stacked chloroplast lamellae. It is also possible, however, that the press treatment produced inside-out vesicles, thereby exposing the inner thylakoid surface to the polymers (see below).

In order to specifically investigate the lateral heterogeneity of charged groups, destacking of chloroplast lamellae under low salt conditions was performed. In that way a lateral movement of membrane constituents takes place resulting in a randomization of thylakoid membrane constituents [13]. Thus, press treatment of destacked chloroplasts yields vesicles with both partition region and stroma lamellae region characteristics. Vesicles of this type were prepared and to reduce the possibility of restacking only small vesicles (Ls100K) were investigated. The cross-partition results showed that the isoelectric point of these randomized membrane fragments was 0.3 pH unit lower (pH 4.4 versus pH 4.7) than for membranes originating exclusively from the stroma lamellae (100K). Considering that the stroma lamellae comprises about 40% of the total chloroplast lamellae [14] an isoelectric point around pH 4.2 was estimated for the partition regions of grana assuming a linear relationship between the cross-point and the amount of membrane material.

The recent observation that Yeda press treatment of chloroplast lamellae produces a fraction of inside-out thylakoid vesicles which can be partly separated from right-side-out material [10,11] allows a direct characterization of the inner thylakoid surface. In order to obtain information about the transverse heterogeneity of charged groups across the thylakoid membrane cross-partition of inside-out vesicles (B) and the right-side-out material (T) was performed. As can be seen in Fig. 1 a low isoelectric point (pH 4.1) was observed for the inside-out fraction while an isoelectric point at 4.5 was observed for the material of normal orientation. Since the B fraction is contaminated by some right-side-out material [10,11] the isoelectric point of the inner thylakoid

surface was estimated to be around pH 4.0 under the same assumption as earlier.

Discussion

For 20 proteins it has been shown that the cross-point agrees well with the isoelectric point obtained by other methods [5,6]. For membranes, fewer cases have been analyzed so far but Ericson [7] could show that for rat liver mitochondria and for two different types of mitochondrial membrane fractions there was a good agreement between the cross-point and the isoelectric point as determined by isoelectric focusing. We therefore assume that cross-partition can be used for the determination of the isoelectric point of cell organelles and membrane vesicles too.

For chloroplast membranes only a few efforts have been made to determine the isoelectric point. In an early study by free flow electrophoresis of Nitella chloroplasts Mercer et al. [15] reported an isoelectric point of 4.2. From measurement on light scattering of spinach chloroplast as a function of pH Dilley and Rothstein [16] found a maximum at pH 4.7 which they suggested to be the isoelectric point of the chloroplast. The cross-point at 4.7 for stacked thylakoids obtained in the present study confirm their indirect measurements.

As can be seen in Fig. 1 the cross-points of various subchloroplast fractions, representing different surface regions of the thylakoid membranes, ranged from pH 4.1 up to 4.7. These values indicate that the various thylakoid surfaces have different isoelectric points as illustrated in the schematic graph of Fig. 2, which for comparison also include the cross-point obtained for the chloroplast envelope (Westrin, H. and Albertsson, P.-Å., unpublished observation). Since the cross-point of a membrane surface reflects a mean value of the isoelectric point of a great number of charged groups, the observed cross-points demonstrate large differences in charge composition between the different surface regions of the thylakoid membrane system. Thus, the observed differences in cross-points between the various subchloroplast fractions demonstrate a charge heterogeneity between both the stroma lamellae surface and the partitions of the stacked grana and between the outer and inner grana thylakoid surfaces. Such heterogeneities reflect differences in the ratio between acidic and basic

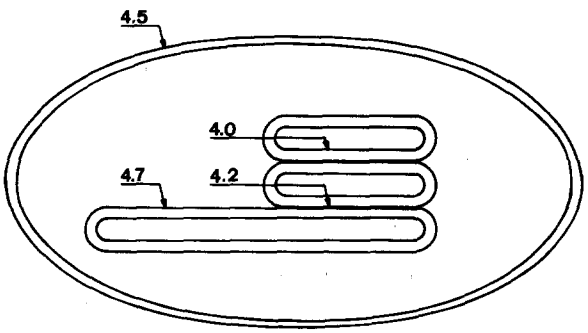

Fig. 2. A schematic graph including estimated isoelectric points for different surface regions of the chloroplast membranes.

amino acids of proteins exposed on the surface as well as differences in the ratio between negatively charged and neutral lipids. Also the ratio between protein and lipid should influence the isoelectric point.

It is not possible, however, to specify the kind of charged groups responsible for the differences between various thylakoid surfaces from the obtained cross-points. Analysis of the overall membrane composition of various subchloroplast fractions have shown [17] that there is no large difference in lipid composition or ratio of protein to lipid between the grana and stroma lamellae. In contrast there are pronounced differences in polypeptide patterns between these regions as shown both by sodium dodecyl sulfate (SDS) gel electrophresis [18,19] and isoelectric focusing [20]. Considering these results it is tempting to suggest that the observed lateral charge heterogeneity is related to the protein moiety rather than to the lipid moiety of the membrane. It should be stressed, however, that such differences in the overall membrane composition do not necessarily have to correlate to cross-partition values which specifically reflect the charge of the membrane surface exposed to the surrounding polymers. Moreover, it seems that in some cases only the most exposed charges of a membrane will influence its partition behaviour. This has recently been shown for phosphatidylcholine liposomes where the phase system only recognizes the trimethylamino group and not the phosphate group [21].

Differences in charged groups along the thylakoid membrane have been suggested to be involved in the stacking behaviour of normal chloroplasts [1,2]. Anderson [1] postulated that grana membranes become stacked as a result of cross-linking by Mg^{2+} between negatively charged groups on adjacent grana membrane surfaces. Negatively charged lipids and negatively charged amino acids would contribute to such negative groups in the partition region, while the unstacked stroma lamellae would be dominated by zwitterionic phospholipids and neutral and basic amino acids. Such a heterogeneous arrangement of charged surface groups should result in a low isoelectric point for the grana partitions compared to the stroma lamellae surface in agreement with the present cross-partition results. The cross-point at pH 4.7 for the stroma lamellae vesicles shows, however that this membrane surface must be negatively charged at physiological pH. Provided that stacking is caused by interlinkage of Mg^{2+} between negative surface charges, also stroma lamellae should stack if not a more specific bridging mechanism excist and/or that other forces, like hydrophobic interactions, are involved.

Berg et al. [2] showed that esterification of thylakoid carboxyl groups induced stacking of stroma lamellae indicating that this membrane region is rich in negatively charged carboxyl groups, preventing membrane pairing under normal conditions. If this implies that the partition region is poor in carboxyl groups its low cross-point would indicate the presence of very acidic groups (like sulpholipids) in combination with a low influence of alkaline groups.

Electron micrographs of chloroplasts near their isoelectric point (pH 4.7) show that also the stroma lamellae form partitions in a similar way as after the carboxyl group modification (Fig. 3). This can be explained by a decrease in the electrostatic repulsion between adjacent stroma lamellae membranes close to their isoelectric point. Another striking feature induced by the low pH is the

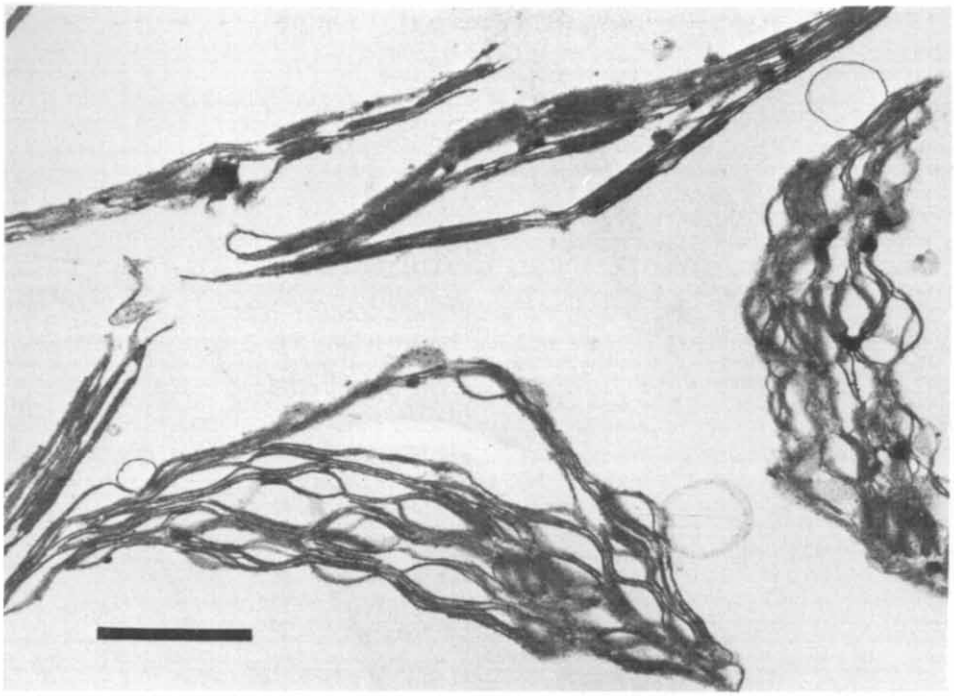

Fig. 3. Electron micrograph of chloroplasts from a phase system at pH 4.7. The sample was fixed in 2% glutaraldehyde, washed with buffer and postfixed in 2% OsO_4 for 2 h. The dehydration was performed stepwise in ethanol and further in propylene oxide before embedding in Epon. Sections were cut and finally stained with uranyl acetate and lead citrate. Bar represents 1 μM.

reduction of the intrathylakoid space. These effects on the ultrastructural appearance of the chloroplast may explain the maximum in light scattering near pH 4.7 observed by Dilley and Rothstein [16].

Characterization of the inner thylakoid surface has become possible after the recently developed procedure for isolation of inside-out thylakoid vesicles. Cross-partition of such inside-out vesicles reveals an asymmetric distribution of charged groups between the outer and inner grana thylakoid surfaces with the latter being more acidic. The low isoelectric point of the grana thylakoid inner surface might be an adaptation to the low pH created in the intrathylakoid space during photosynthesis. With such a low isoelectric point this membrane remains negatively charged even down to around pH 4.

Although the present study shows that there is a surface charge heterogeneity along and across the chloroplast lamellae further studies have to be performed in order to elucidate in more detail the surface characteristics of the different membrane regions.

Acknowledgement

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