TRYPSINATION OF INSIDE-OUT CHLOROPLAST THYLAKOID VESICLES FOR LOCALIZATION OF THE WATER-SPLITTING SITE

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1. Introduction

Many conflicting results have been reported concerning the location of the water splitting site in the chloroplast thylakoid membrane [1,2]. An internal location has been assumed mainly from the internal release of protons linked to oxygen production [3,4] and the selective release of manganese into the lumenal space upon Tris treatment [5]. In contrast, an external exposure has been inferred from the inactivation of photosystem II electron transport by chemical probes considered to be unable to penetrate the membrane [6,7]. A location at the outer surface has also been concluded from the trypsin digestion of the chloroplast lamellae [8,9] while the effect of trypsin has been claimed to be due mainly to proteolysis of the reducing side of photosystem II [10,11].

These conflicting results may reflect the inherent limitations in modifying only the outer membrane surface for studies on the transverse organization of a membrane. Modification of a component on the outer surface may cause rearrangements in the membrane which leads to inactivation of internal catalytic sites. However, the absence of effects does not reveal whether a component is located internally or just shielded behind some bulky membrane constituents. A direct comparison between the effects of non-penetrating reagents on the outer and inner membrane surfaces is therefore desirable. Such a comparison has been possible for the chloroplast thylakoid membrane for the first time by the recent isolation of inside-out vesicles by two-phase partition [12–15]. Their reversed sidedness has been demonstrated by the direction of the proton and electrical gradients [12–14] and by freeze-fracture electron microscopy [15].

Here the trypsin effect on photosystem II electron transport has been compared for closed thylakoid membranes of opposite sidedness. The results show that DCIP reduction using water as electron donor was sensitive to trypsin in both types of membranes. However, upon addition of diphenyl carbazide the activity was completely restored in the inside-out but not in the rightside-out fraction. This strongly favours an internal location of the water splitting site of the photosynthetic electron transport.

Abbreviations: chl, chlorophyll; DCIP, 2,6-dichlorophenol indophenol; DPC, 1,5-diphenyl carbazide

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2. Materials and methods

2.1. *Preparation of thylakoid membrane fractions*

Stacked spinach chloroplast lamellae (class II) [16] were fragmented by a Yeda press disintegration procedure [13]. Inside-out thylakoid vesicles formed during this procedure were separated from rightside-out material by partition in an aqueous dextran–polyethylene glycol two-phase system [13]. Chloroplast fragments (5 ml at 800 μg chl/ml) were added to 20 g polymer mixture to yield the following composition: 5.7% (w/w) dextran 500, 5.7% (w/w) polyethylene glycol 4000, 10 mmol/kg sodium phosphate buffer (pH 7.4), 5 mmol/kg NaCl, 20 mmol/kg sucrose and chloroplast material corresponding to 4 mg chl. The concentrations are based on the final weight of the

phase system. The phase system was carefully mixed and allowed to separate. The top phase (T1) and bottom phase (B1) were collected and repartitioned with pure bottom phase and top phase, respectively, yielding fractions T2 and B2. The B2 fraction was purified by a further partition step giving a B3 fraction. The material in this fraction was removed from the viscous bottom phase by a final partition step with a top phase containing the positively charged trimethylamino-polyethylene glycol [17]. The B3 fraction contains the inside-out thylakoid vesicles while the T2 fraction contains vesicles of normal sidedness.

Rightside-out fraction destacked chloroplasts were mainly used. They were prepared from stacked chloroplast lamellae [16] by incubation for $\geqslant 30$ min in 50 mM tricine (pH 7.4), 100 mM sucrose. Destacked chloroplasts were preferred since the partitions might restrict the enzyme action.

2.2. *Trypsin treatment*

Prior to trypsination all samples were set at 35 μg chl/ml using the top phase from a system containing 5.7% (w/w) dextran 500, 5.7% (w/w) polyethylene glycol 4000, 50 mmol/kg tricine (pH 7.4) and 100 mmol/kg sucrose. Trypsination was performed at room temperature with a trypsin (Sigma type XI): chlorophyll ratio of 0.025:2. After 2.5 min digestion under magnetic stirring, soybean trypsin inhibitor (Sigma type I-S) was added to yield an inhibitor to trypsin ratio of 100. The control samples of each fraction received a mixture of trypsin and inhibitor yielding the same final concentration as for the digested samples.

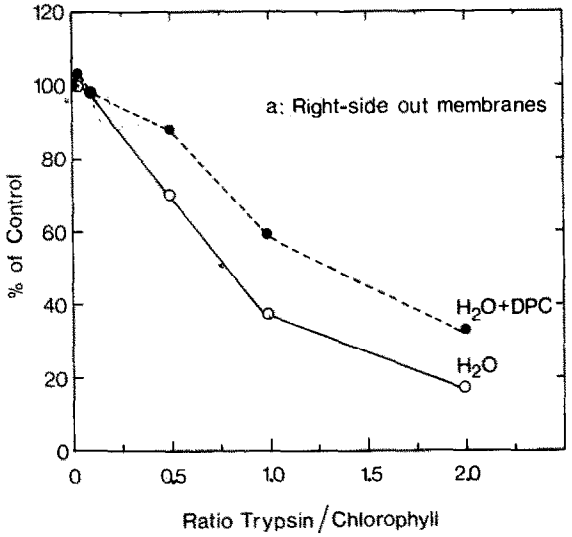
2.3. *Electron transport measurement*

Photosystem II electron transport activities with DCIP as electron acceptor was followed at 550 nm using an Aminco DW-2 spectrophotometer operating in split beam mode at 20°C. The intensity of the actinic light ($\lambda > 610$ nm) was 160 W/m^2. As electron donors both water and diphenyl carbazide were used. The reaction mixtures are given in the legend to fig.1.

3. Results

The influence of trypsin on the rate of photosystem II electron transport, measured as DCIP reduction, in the unbroken chloroplast lamellae and the inside-out fraction is shown in fig.1a and 1b, respectively. With water as electron donor a pronounced inhibition was observed both for unbroken chloroplast lamellae and inside-out thylakoid vesicles (B3). On addition of diphenyl carbazide the photosystem II activity of the inside-out vesicles was completely restored (fig.1b). In contrast, diphenyl carbazide had only a minor effect on the activity of the unbroken chloroplast lamellae (fig.1a). The same inhibitory pattern both in the presence and absence of diphenyl carbazide, as demonstrated for the unbroken chloroplast lamellae (fig.1a), was obtained for thylakoid vesicles of normal sidedness (T2, not shown). This result indicates that the differences in the effects of trypsin on rightside-out and inside-out material are due to differences in sidedness rather than size. The stimulation by diphenyl carbazide in the T2 fraction was slightly stronger than for chloroplast lamellae, which can be explained by the contamination of inside-out vesicles (10%) in the T2 fraction [15].

The higher control activity found for the inside-out material using diphenyl carbazide + water as electron donors, compared to the unbroken chloroplast lamellae, is in agreement with our previous finding that these vesicles are enriched in photosystem II [16]. Due to the Yeda press treatment



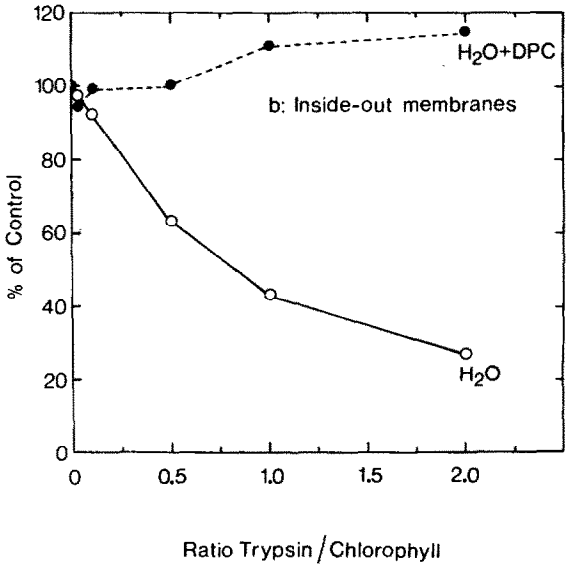


Fig.1. Effects of trypsin on DCIP reduction in chloroplast thylakoid membranes of opposite sidedness. The ΔA_{550} was measured in a suspension (pH 6.1) of the following composition: 40 mM sodium phosphate; 4.4 mM NaCl; 22 mM tricine; 44 mM sucrose; 36 μM DCIP; chloroplast material corresponding to 6.7 μg chl/ml and, where indicated, 1.1 mM diphenyl carbazide. The 100% rates for unbroken chloroplast lamellae were 156 μmol DCIP reduced .mg chl^{-1} .h^{-1} both in the absence and presence of diphenyl carbazide and for the inside-out membranes 137 and 264 μmol .mg chl^{-1} .h^{-1} in the absence and presence of diphenyl carbazide, respectively.

part of their water splitting activity is lost, which explains why diphenyl carbazide stimulated electron transport in the control samples. In the presence of diphenyl carbazide not only a total restoration but also a small stimulation of the DCIP reduction was observed for the inside-out fraction after trypsin treatment (fig.1b). This might reflect an increased accessibility of diphenyl carbazide to the tryptic-digested surface.

4. Discussion

The isolation of inside-out vesicles allows for the first time a direct comparison of proteolytic effects on the outer and inner thylakoid surfaces. The trypsination of the inside-out vesicles resulted in loss of photosystem II electron transport which could be fully restored by addition of diphenyl carbazide. This was not the case for thylakoid membranes of normal sidedness. These results strongly suggest that the water-splitting site is located at the inner thylakoid surface.

The absence of any effect of trypsin on the reaction from diphenyl carbazide to DCIP in the inside-out fraction indicates that this reaction sequence is not exposed to the inner surface. In fact an external location is suggested since this electron transport path is affected in rightside-out thylakoids. Whether this trypsin block is on the donor side (between diphenyl carbazide and P680) or on the acceptor side can not be judged at present. Studies are in progress to solve this question.

A minor effect on the water-splitting reaction of trypsin from the outside can not be excluded since a small stimulatory effect of diphenyl carbazide was found for the rightside-out material. This stimulation might be due to secondary effects as suggested [18].

For the unbroken chloroplast lamellae the effects of trypsin on the reaction from water to DCIP were similar to those reported [8,9]. The small stimulation on addition of an artificial electron donor for photosystem II, such as diphenyl carbazide or tetraphenyl boron, is in agreement with [8,10] but not with [9] where total reactivation was reported.

Concerning the tryptic effect on the water-splitting activity from the inner surface, our results contradict [9]. On sonication of *Chlamydomonas reinhardi* chloroplast vesicles in the presence of trypsin, followed by addition of trypsin inhibitor, they observed no deleterious effects on the water-splitting system or any other electron transport activities measured. In our opinion it is difficult to compare the effect of trypsin when, on one hand, it is added externally and on the other hand, trapped inside vesicles, since it is hard to control the amount actually trapped. Therefore, direct comparison between inside-out and rightside-out membranes is a preferable approach.

In conclusion, our findings argue strongly for a model where the water-splitting system is exposed at the inner thylakoid surface, while an external location is suggested for a part of the pathway between diphenyl carbazide and DCIP.

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References

[1] Trebst, A. (1974) Ann. Rev. Plant. Physiol. 25, 423–458.
[2] Diner, B. A. and Joliot, P. (1977) in: Encyclopedia of Plant Physiology (Trebst, A. and Avron, M. eds) vol. 5, pp. 187–205, Springer-Verlag, Berlin.
[3] Fowler, C. F. and Kok, B. (1974) Biochim. Biophys. Acta 357, 299–307.
[4] Ausländer, W. and Junge, W. (1975) FEBS Lett. 59, 310–315.
[5] Blankenship, R. E. and Sauer, K. (1974) Biochim. Biophys. Acta 357, 252–266.
[6] Giaquinta, R. T., Dilley, R. A. and Anderson, B. J. (1973) Biochem. Biophys. Res. Commun. 52, 1410–1417.
[7] Arntzen, C. J., Vernotte, C., Briantais, J. M. and Armond, P. (1974) Biochim. Biophys. Acta 368, 39–53.
[8] Selman, B. R. and Bannister, T. T. (1971) Biochim. Biophys. Acta 253, 428–436.
[9] Regitz, G. and Ohad, I. (1976) J. Biol. Chem. 251, 247–252.
[10] Renger, G., Erixon, K., Döring, G. and Wolff, Ch. (1976) Biochim. Biophys. Acta 440, 278–286.
[11] Renger, G. (1976) Biochim. Biophys. Acta 440, 287–300.
[12] Andersson, B., Åkerlund, H.-E. and Albertsson, P.-Å. (1977) FEBS Lett. 77, 141–145.
[13] Andersson, B. and Åkerlund, H.-E. (1978) Biochim. Biophys. Acta 503, 462–472.
[14] Gräber, P., Zickler, A. and Åkerlund, H.-E. (1978) FEBS Lett. 96, 233–237.
[15] Andersson, B., Simpson, D. J. and Høyer-Hansen, G. (1978) Carlsberg Res. Commun. 43, 77–89.
[16] Åkerlund, H.-E., Andersson, B. and Albertsson, P.-Å. (1976) Biochim. Biophys. Acta 449, 525–535.
[17] Johansson, G., Hartman, A. and Albertsson, P.-Å. (1973) Eur. J. Biochem. 33, 379–386.
[18] Renger, G. and Tieman, R. (1979) Biochim. Biophys. Acta 545, 316–324.