# Effect of α-, β- and γ-Cyclodextrins on Oxygen Evolution by the Thylakoid Membrane. Influence of pH and Temperature

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The present work investigates the effect of α-, β- and γ-cyclodextrins (CD), i.e., α-CD, β-CD and γ-CD, on the oxygen evolution activity, the protein content and the uv-vis spectroscopic characteristics of thylakoid membranes. To study the pH-dependence, the thylakoids were incubated with the cyclodextrins at 273 K for a period of 10 min in the pH range from 5.5 to 9.0. To study the temperature-dependence the membranes were incubated at 273 and 293 K at pH 6.5, that is, the pH which induces a maximal oxygen evolution in the thylakoid preparations. The major observations are: (*i*) a stimulation of oxygen evolution in thylakoids incubated with α- and β-CD either in acidic or alkaline conditions, (*ii*) a low inhibitory effect induced by γ-CD on oxygen evolution, (*iii*) a significant decrease of the stimulatory effect of α- and β-CD on oxygen evolution as the incubation temperature is raised from 273 to 293 K, (*iv*) the apparent inability of the cyclodextrins to change the protein contents of the thylakoids, and (*v*) a significant CD-induced red-shift from 681 to 683 nm observed in the absorption and second derivative spectra of the thylakoid membranes treated with β-CD. First, it was found that the temperature effect described here is in accord with the general trend of the chemical effect of various cyclodextrins, i.e., the increase of the CD efficiency with decreasing temperature. Secondly, the CD effect is related to the size of the inner cavity diameter of the cyclodextrin molecules. An important conclusion in this work is that the molecular targets of the cyclodextrins are not limited to the thylakoid lipids as was described previously [Rawyler A. and Siegenthaler P.A. (1996) Biochim. Biophys. Acta **1278**, 89–97], but are located as well in other molecular species exposed at the stromal side of the thylakoid membrane. In particular, the CD-induced red-shift from 681 to 683 nm in the absorption and second derivative spectra of the thylakoid membranes indicates that the cyclodextrins targets might be either the exposed heme macrocycle in cytochrome b559, or the chlorophylls and pheophytins in the pigment-proteins of the photosystems I and II.

## Introduction

The electron transport properties of intact thylakoid membranes as well as their fluorescence characteristics at 77 and 293 K were shown to be altered by cyclodextrins (Rawyler and Siegenthaler, 1996), a class of cyclic oligomeric amylose compounds constituted of 6 to 8 units of D-glucose linked by α-1,4 bonds (Li and Purdy, 1992; D'Souza and Lipkowitz, 1998). Rawyler and Siegenthaler (1996) interpreted the cyclodextrin (CD) effect as a perturbation of the excitation energy distribution between the photosystem II (PSII) and the photosystem I (PSI) macromolecular complexes. They concluded moreover that the observed effect might have resulted from structural modifications in the thylakoid membrane following the partial depletion of its lipid content upon a cyclodextrin treatment. This conclusion brings about two major questions. The first concerns the polypeptides of the thylakoid membrane

*Abbreviations:* α-CD, β-CD, γ-CD, α-, β- and γ-cyclodextrin; Chl, chlorophyll; DGDG, digalactosyldiacylglycerol; HEPES, N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]; MES, 2-[N-morpholino]ethanesulfonic acid; MGDG, monogalactosyldiacylglycerol; PG, phosphatidylglycerol; PSI, photosystem I; PSII, photosystem II; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; SQDG, sulfoquinovosyldiacylglycerol; Tricine, N-tris[hydroxymethyl]-methylglycine.

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whose composition is far more complex than the simple lipid content of the thylakoids (see, e.g., Murata *et al.*, 1990, Barber *et al.*, 1999, Zouni *et al.*, 2001). The second question is related to the chemical structure and geometry of the various cyclodextrins (see, e.g., Li and Purdy, 1992) which are particularly well adapted to make the various surface protein components of the granal thylakoids their potential targets. In this perspective, we extended in the present work the study of the CD effect to the analysis of the function of the oxygen evolving complex of PSII in intact thylakoid membranes with the aim of clarifying further the cyclodextrin-induced changes described in Rawyler and Siegenthaler (1996).

The cyclodextrins have a structure resembling a truncated cone geometry or torus shape (Li and Purdy, 1992; D'Souza and Lipkowitz, 1998) with a narrower- (nR) and a wider-rim (wR) side, or diameter (Fig. 1). The primary 6-hydroxyl groups are on the nR side of the molecule, and the secondary 2- and 3-hydroxyl groups on the wR side. The most common cyclodextrins are the α-, β- and γ-CD which contain, respectively, 6, 7 and 8 glucose units (hexa-, hepta- and octo-amylose) with outer diameters in the wR side of respectively 1.37, 1.53 and 1.69 nm (cf. Fig. 3 of Li and Purdy, 1992). It was found that the variable number of glucose units in the cyclodextrins structure influences their chemical properties on account of the different size of their inner diameter in the wR side, i.e., 0.57 nm (α-CD), 0.78 nm (β-CD) and 0.95 nm (γ-CD) (Li and Purdy, 1992). Moreover, the geometrical arrangement of the glucose residues in the cyclodextrin molecules exposes a large number of hydroxyl groups on the CD external surface which is therefore hydrophilic (Fig. 1), whereas the distribution of non-bonding electron pairs of the glycosidic oxygen bridges makes the internal cavity hydrophobic, or at least less polar than the external surface (Li and Purdy, 1992). This apolar nature of the inner cavity facilitates the cyclodextrins binding to the hydrophobic moiety of neutral or ionic molecules of small size (Li and Purdy, 1992; D'Souza and Lipkowitz, 1998). A second fundamental condition for the formation of a viable guest-host inclusion complex is the size and shape of the guest species as is discussed in the studies by D'Souza and Lipkowitz (1998), Gu *et al.* (1999, 2000), Manunza *et al.* (1997) and Gu and Bayley (2000). These authors identified successfully the properties of several kinds of ligand molecules which are capable of being included, or encapsulated into the internal cavity of the cyclodextrins.

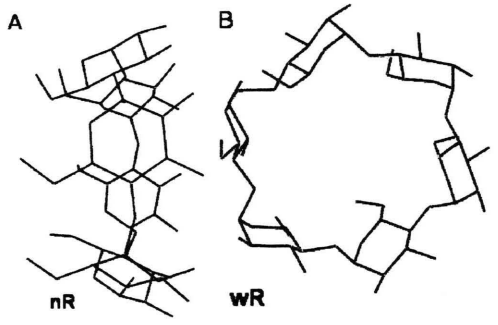


Fig. 1. Lateral (A) and front (B) views of α-cyclodextrin (α-CD) obtained from x-ray diffraction analysis (Mikami *et al.*, 1992) of its complex with β-amylase (α-1, 4-glucan maltohydrolase, 1btc.pdb) obtained from the Protein Data Bank (PDB) (Bernstein *et al.*, 1977; Berman *et al.*, 2000). The PDB access number of the β-amylase/α-CD complex is 1btc.pdb. α-CD is a hexa-amylose consisting of six D-glucose units linked together by α-1,4 bonds arranged in a truncated cone geometry with a narrower- (nR) and a wider-rim (wR) side. The inner and outer diameters in the wR side are 0.57 and 1.37 nm, respectively.

The general characteristics of the cyclodextrin effect are first the interaction of the hydrophobic moiety of the guest molecule with the functional groups in the cyclodextrin cavity, then the hydrogen bonding coupling between the polar residues of guest molecules and the hydroxyls of the cyclodextrin. The interaction of guest molecules with the cyclodextrins alters many chemical and physiological properties such as the protection against oxidation and photolysis, thereby causing the modification of chemical reactivity and several biological properties (Li and Purdy, 1992). The cyclodextrins were also found to have the interesting property of inducing significant modifications in the uv-vis absorption spectra of the guest compounds (Li and Purdy, 1992). This effect is used in the present work for studying the pigment-protein complexes of the thylakoid membrane.

Another important aspect of the cyclodextrin effect is the sensitivity of the guest-host inclusion complex to temperature; that is, a stability decrease of the inclusion complex with increasing temperature (Li and Purdy, 1992). To explain this temperature-dependence, a thermodynamic study

on the hydrogen bonding and hydrophobic interactions in the guest-host inclusion complexes of cyclodextrins was attempted by Ross and Rekharsky (1996). The major finding is the decrease of the hydrogen bond stability with increasing temperature (i.e., 'hydrogen-bond melting'), in contrast with the temperature effect on the hydrophobic interaction whose stability was shown to remain essentially constant.

The present study investigates the effect of the α-, β- and γ-cyclodextrins on the oxygen evolution activity, the protein composition and the spectral characteristics of intact thylakoids. In this respect, it is noted that this is the first work using γ-CD in studies of the functional and structural aspects of the photosynthetic membrane. Hereunder, we shall discuss the correlation of the pH- and temperature-dependence of the CD effect to structural changes in the stromal side of the thylakoid membrane.

## Materials and Methods

### *Chemicals*

Sodium dodecylsulfate, acrylamide, α- and β-cyclodextrins were purchased from Sigma Chemical Company (St. Louis, MO). Dichlorobenzoquinone was from Pfaltz and Bauer (Waterbury, CT), and γ-cyclodextrin was purchased from Fluka-Chemie (Buchs, Switzerland). The Formvar/carbon coated copper grids (400 mesh size) and the glutaraldehyde used for electron microscopy were obtained from Mecalab Ltd. (Montreal, Canada). All other chemicals including uranyl acetate were from Fisher Scientific Company (Fair Lawn, NJ).

### *Preparation of the thylakoid membranes*

Thylakoid membranes were obtained from the chloroplasts of 6–8 day old barley seedlings (*Hordeum vulgare*) according to the procedure described in Berthold *et al.* (1981) with some modifications. Barley leaves were homogenized at 273 K in a medium containing 50 mM Tricine (N-tris[hydroxymethyl]-methylglycine)-NaOH (pH 7.8), 400 mM sorbitol, 10 mM NaCl and 5 mM $MgCl_2$ (buffer A), and filtered through eight layers of cheesecloth. The filtrate was centrifuged at 1000 x *g* for 5 min at 277 K and the resultant pellet (chloroplasts) was centrifuged in the same conditions in buffer A. The thylakoids were obtained by suspending the chloroplasts pellet in a buffer containing 50 mM Tricine-NaOH (pH 7.8), 10 mM NaCl and 5 mM $MgCl_2$ (buffer B), followed by a centrifugation at 1000 x *g* for 5 min at 277 K. The precipitate (thylakoid membranes) was suspended in a buffer containing 20 mM MES (2-[N-morpholino]ethanesulfonic acid)-NaOH (pH 6.5), 400 mM sucrose, 15 mM NaCl and 5 mM $MgCl_2$ (buffer C), and centrifuged at 1000 x *g* for 5 min at 277 K. The final precipitate was suspended in buffer C to give a chlorophyll concentration of 2 mg/ml. The chlorophyll concentration of the membranes was estimated using 80% (v/v) acetone solutions according to the procedure of Arnon (1949). These thylakoid membrane preparations were stored at 143 K until use.

### *Treatment of the thylakoids with cyclodextrins*

For pH treatments, aliquots of thylakoid membranes (50 μg Chl/ml) were suspended in a series of three buffers to cover the pH range from 5.5 to 9.0. That is, 20 mM MES (pH 5.5–6.5), 20 mM HEPES (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]) (pH 7.0 and 7.5) and 20 mM Tricine (pH 8.0–9.0) which contained, in addition, 400 mM sorbitol. The samples were incubated with 10 mg/ml of α-, β- and γ-cyclodextrins at 273 or 293 K for 10 min in the dark, and then centrifuged immediately at 8000 x *g* for 5 min at 277 K to precipitate the thylakoid membranes. These membranes were dispersed in the buffer used for measurement of oxygen evolution which contained 20 mM MES-NaOH (pH 6.5), 400 mM sucrose, 15 mM NaCl and 5 mM $MgCl_2$.

For temperature-dependence treatment, aliquots of thylakoid membranes (50 μg Chl/ml) were suspended in a 20 mM MES buffer, pH 6.5, which contained 400 mM sorbitol. The samples were incubated with 10 mg/ml of α-, β- and γ-cyclodextrins at 273 or 293 K for 10 min in the dark, and then centrifuged immediately at 8000 x *g* for 5 min at 277 K to precipitate the thylakoid membranes. These membranes were dispersed in the buffer used for measurement of oxygen evolution which contained 20 mM MES-NaOH (pH 6.5), 400 mM sucrose, 15 mM NaCl and 5 mM $MgCl_2$.

Oxygen evolution measurements were made with a Hansatech Oxygen Electrode at 298 K. The

chlorophyll concentration in the reaction mixture was 12.5 $\mu$g/ml. DCBQ (350 $\mu$M) was used as electron acceptor. The thylakoid preparations were irradiated with white light at saturating light intensity.

*Polyacrylamide gel electrophoresis*

The polypeptide composition of the untreated thylakoids and the thylakoids treated with cyclodextrins was analysed by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Chua, 1980). The standard proteins and the thylakoid membrane polypeptides were resolved on a linear gradient gel as described by Nénoné *et al.* (1998). Before loading into the gel slots, the thylakoid membrane samples were first solubilized with SDS. This was followed by a centrifugation at 13600 x *g* for 5 min to remove the unsolubilized membranes. Upon electrophoresis, the gels were stained for three hours with a solution containing 50% methanol, 10% acetic acid and 0.125% R-250 Coomassie brilliant blue (Bio-Rad Laboratories, Richmond, CA). Gel destaining was performed in a solution containing 60% water, 30% methanol and 10% acetic acid. To estimate the molecular mass of the proteins (MW) a plot of log MW *vs.* migrated distance (d) in cm (log MW *vs.* d) was used for the unknown proteins and the protein markers. The molecular masses of the markers (RPN 800 kit from Amersham International plc, Buckinghamshire, England) were 15, 25, 30, 35, 50, 75, 105, 160 and 250 kDa.

*Transmission electron microscopy*

The integrity of the thylakoid membranes before and after the cyclodextrin treatment was checked in a Philips transmission electron microscope (Eindhoven, The Netherlands), model EM208S. For this purpose, the thylakoid membranes were first fixed with 2.5% glutaraldehyde for 30 min, then one drop of the preparations (200 $\mu$g Chl./ml) was loaded on the Formvar/carbon coated copper grids (400 mesh size) and negatively stained with 1% uranyl acetate for a few seconds. These negatively stained preparations were used for the analysis of the thylakoids shape and size. The images were obtained with a model 1.6i Kodak Megagraph (Rochester, NY), and analysed with the analySIS 3.0 software from Soft Imaging System Gmbh (Münster, Germany).

The electron microscopy images showed systematically that the thylakoid particles were circular in shape. Their calculated average diameter was found to be 454 ± 45 nm in good agreement with published data (see, e.g., Weier *et al.*, 1967). Moreover, the electron microscopy analysis indicated clearly that the thylakoid membranes used in this work did not present any visible sign of structural damage.

*Other methods*

The atomic coordinates of α-cyclodextrin were those in the complex between β-amylase (α-1,4-glucan maltohydrolase, EC 3.2.1.2) and α-CD (Mikami *et al.*, 1992) obtained from the Protein Data Bank (PDB) (Bernstein *et al.*, 1977; Berman *et al.*, 2000). The PDB access number of the β-amylase/α-CD complex is 1btc.pdb. The atomic distances and other molecular details were determined with the WebLab ViewerPro software from Molecular Simulations Inc. (San Diego, CA).

## Results and Discussion

*pH effect on oxygen evolution in cyclodextrin-treated thylakoids*

The effect of the incubation media pH (5.5 to 9.0) on the oxygen evolving activity in thylakoid membranes treated with α-, β- and γ-CD, and in a control medium which did not contain any cyclodextrin, is presented in Fig. 2. To eliminate the cyclodextrin molecules that were not bound to the thylakoid membrane constituents (free CD), the thylakoid samples were first precipitated by centrifugation and then suspended in the reaction medium buffer at pH 6.5 before the measurement of oxygen evolution (see Materials and Methods). Fig. 2 shows that in the control thylakoid samples the oxygen evolution increases steadily in the acidic side of the pH scale up to about pH 6.5 where a maximum is observed, then decreases in the alkaline side of the pH scale. One sees that the maximal oxygen evolution activity observed at pH 6.5, i.e., 172 ± 12.1 μmol $O_2$/mgChl × h, is reduced to 112 ± 4.3 μmol $O_2$/mgChl × h, that is, a decrease of about 35%, in the thylakoid preparations incubated at pH 9.0. It is noted that this pH-depen-

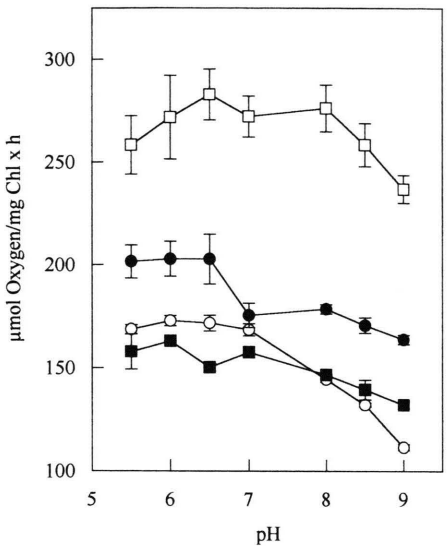


Fig. 2. pH-dependence of oxygen evolution in control (untreated) thylakoid membranes (○) and in membranes treated with alpha-CD (●), beta-CD (□) and gamma-CD (■). *Abbreviations*: Chl, chlorophyll; CD, cyclodextrin.

dence of oxygen evolution in the control samples (Fig. 2) is in accord with the pH-dependence observed previously in thylakoid membranes or in isolated photosystem II particles (see Nénonéne and Fragata, 1990, and references therein). This trend in the oxygen evolution inactivation in the alkaline media was attributed to irreversible conformational changes of the PSII polypeptides or to inadequate arrangements of the lipid-protein complexes.

Fig. 2 reveals also that β-CD presents the strongest enhancement effect on the oxygen-evolving activity of the thylakoids. It is seen that at about pH 6.5 the oxygen evolution in the β-CD-treated thylakoid membranes increases to a maximum of 283 ± 24.8 μmol $O_2$/mgChl × h, whereas in the control untreated samples at the same pH the average activity is about 172 ± 12.1 μmol $O_2$/mgChl × h. In addition, Fig. 2 shows that β-CD does not counteract completely the loss of oxygen evolving activity at pH's higher than 6.5, thus following the trend of the control samples, but can nevertheless sustain oxygen evolution rates much higher than the ones observed in the untreated control samples. At pH 9.0, the activity of thylakoids is 112 ± 4.3 μmol $O_2$/mgChl × h in the control samples, whereas the β-CD-treated samples display an oxygen-evolving activity of 237 ± 13.6 μmol $O_2$/mgChl × h, that is, a two fold increase over the control samples which is much higher than the observed maximum activity of the control at pH 6.5.

In addition, Fig. 2 shows that the pH effect observed in β-CD-treated samples is also seen in thylakoid preparations incubated with α-CD in the same experimental conditions. That is, α-CD enhances as well the oxygen evolution in the thylakoids as a function of pH but to a much lesser extent. In short, one sees in Fig. 2 that α-CD gives rise to a 17% increase of the oxygen evolution at pH 6.5 in relation to the untreated thylakoids, while the oxygen evolving activity is inactivated by about 20% at pH 9.0. Interestingly, the γ-cyclodextrin does not seem to enhance the activity of oxygen evolution in the thylakoid membrane in the pH range from 5.5 to 9.0, but appears to have instead a tendency to stabilize the oxygen-evolving activity in the PSII complex.

In summary, the data in Fig. 2 reveal what appears to be a similar mode of action of α- and β-CD, that is, an about identical trend of the oxygen evolution enhancement up to pH 6.5 on the one hand, and the protection of the oxygen evolving complex in the alkaline side of pH on the other hand. The α- and β-CD capacity to protect the function of photosystem II complex against the deleterious effects of high alkaline pH presents however considerable differences. In fact, this study shows that the oxygen evolution in thylakoid membranes treated with α-CD at pH 6.5 or 9.0 is approximately half the one observed in β-CD-treated samples.

### *Temperature effect on oxygen evolution in cyclodextrin-treated thylakoids*

It was observed in several instances (see, e.g., Li and Purdy, 1992) that the activity of the cyclodextrin molecules is influenced by temperature, that is, the cyclodextrins were shown to be in general more efficient at lower temperatures on account of the decreased stability of the CD inclusion complex as the temperature increases. The molecular basis of this effect lies in the strong decrease of the hydrogen bond stability with increasing temperature, whereas in similar conditions the stability of the hydrophobic interactions

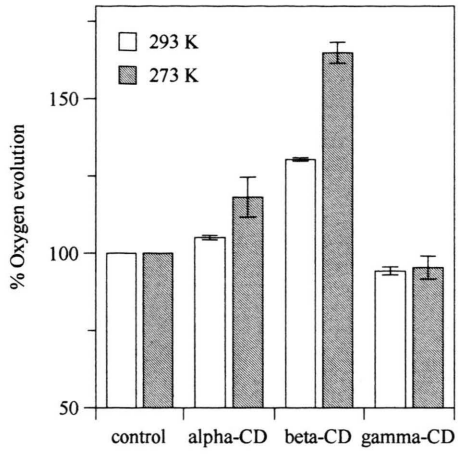


Fig. 3. Temperature-dependence of oxygen evolution in thylakoid membranes treated with α-, β- and γ-cyclodextrins, i.e., α-CD, β-CD and γ-CD, at 273 and 293 K. The average oxygen evolution activities in the control (untreated) samples incubated at 273 and 293 K were respectively 184 ± 12 and 206 ± 13 μmol $O_2$/mgChl × h. *Abbreviations*: Chl, chlorophyll; CD, cyclodextrin.

remains unaffected by temperature (Ross and Rekharsky, 1996). These properties have important practical implications in works on the bio-activity of the cyclodextrins. An interesting example is their utilization in the selective depletion of the thylakoid membrane from several lipid species (Rawyler and Siegenthaler, 1996).

In this work, we studied the influence of temperature (273 and 293 K) on the oxygen evolution in CD-treated thylakoids incubated at pH 6.5, i.e., the pH optimum for the activity of the control untreated samples and the samples treated with α-CD- and β-CD (Fig. 2). It is worth noting that this pH has been often shown to be as well the pH for optimal activity in isolated PSII particles (see, e.g., Nénonéne and Fragata, 1990). We note at first that the oxygen evolution in the control (untreated) samples incubated at 273 and 293 K is respectively 184 ± 12 and 206 ± 13 μmol $O_2$/mg Chl × h (Fig. 3 and Table I). The difference of approximately 12% between the oxygen evolving activities of the thylakoid samples incubated at 273 and 293 K is consistent with earlier observations that the heat-induced effect becomes only important at temperatures higher than 298–303 K (see discussions in, e.g., Joshi and Fragata, 1999).

The results displayed in Fig. 3 (cf. also Table I) show that temperature influences markedly the efficiency of α- and β-CD in controlling the oxygen evolving activity in the thylakoid membrane. At 293 K, the α-CD-treated samples have an oxygen evolving activity quite similar to the one observed in the control (untreated) samples, i.e., 216 ± 16 μmol $O_2$/mg Chl × h as compared to 206 ± 13 μmol $O_2$/mg Chl × h in the control (about 5% increase; cf. Table I), whereas at 273 K the α-CD-

Table I. Effect of α- and β-cyclodextrins on the lipid removal and oxygen evolution activity in the thylakoid membrane[a]

| Cyclodextrin | Lipid removal at 273 K, mol%[b] | | | | Oxygen evolution, μmol$O_2$/mg Chl × h[c] | |
|---|---|---|---|---|---|---|
| | MGDG | DGDG | PG | SQDG | 273 K | 293 K |
| NT[d] | | | | | 184 ± 12 | 206 ± 13 |
| α | 5.4 ± 4.8 | 5.4 ± 2.7 | 39.1 ± 7.4 | 42.4 ± 2.1 | 215 ± 33 (+17 %)[e] | 216 ± 16 (+5 %) |
| β | 13.4 ± 2.4 | 14.5 ± 3.0 | 12.1 ± 2.6 | 8.1 ± 3.5 | 302 ± 17 (+64 %) | 268 ± 18 (+30 %) |

[a] In the lipid removal experiments the thylakoid samples were incubated for 30 min with the cyclodextrins, whereas in the oxygen evolution experiments the incubation time was 10 min; however, Table 4 of Rawyler and Siegenthaler (1996) indicates that upon 10 min incubation with the cyclodextrins the delipidation of the thylakoid samples does not show any significant increase.

[b] Data from Table I in Rawyler and Siegenthaler (1996)

[c] Data from Fig. 3.

[d] *Abbreviations:* α, α-cyclodestrin; β, β-cyclodextrin; DGDG, digalactosyldiacylglycerol; MGDG, monogalactosyldiacylglycerol; NT, control (untreated) thylakoid samples; PG, phosphatidylglycerol; SQDG, sulfoquinovosyldiacylglycerol.

[e] The figures in parenthesis are the % increase in oxygen evolving activity of the thylakoid samples in relation to the control (untreated) samples (NT) incubated at 273 and 293 K.

treated samples exhibit an oxygen-evolving activity of 215 ± 33 μmol $O_2$/mgChl × h which corresponds to an activity enhancement of about 17% in relation to the control samples (184 ± 12 μmol $O_2$/mg Chl × h). A similar trend is observed with β-CD whose effect was shown to be strongly dependent on temperature. In fact, the oxygen-evolving activity at 273 K is 302 ± 17 μmol $O_2$/mgChl × h as compared to 184 ± 12 μmol $O_2$/mgChl × h in the control thylakoid samples, i.e., a 64% increase, whereas at 293 K the enhancement of oxygen-evolving activity by β-CD (268 ± 18 μmol $O_2$/mgChl × h) is only 30% above the oxygen evolution observed in the control thylakoid samples (206 ± 13 μmol $O_2$/mg Chl × h).

It is important to note that the higher level of oxygen evolution observed in thylakoid membranes treated with α- and β-CD at the lower incubation temperature, i.e., 273 K, as compared to the oxygen evolution in CD-treated membranes incubated at 293 K is in close agreement with the data obtained with guest-host complexes constituted of small organic molecules and cyclodextrins where a low temperature activation of the CD effect has been systematically reported (see, e.g., Li and Purdy, 1992). To explain the temperature-dependence of the cyclodextrin effect, Ross and Rekharsky (1996) performed a thermodynamic study on the hydrogen bonding and the hydrophobic interactions in the inclusion complex. On the one hand, Ross and Rekharsky (1996) concluded that the hydrogen bond stability decreases with increasing temperature, an effect which is known as 'hydrogen-bond melting'. They observed, on the other hand, that temperature does not affect the stability of the hydrophobic interaction which was shown to remain essentially constant.

*Polypeptide composition in cyclodextrin-treated thylakoid membranes*

Rawyler and Siegenthaler (1996) presented convincing evidence indicating that some of the cyclodextrins species used in their work affect the lipid composition of the thylakoid membrane. For example, α- and β-CD were shown to deplete the thylakoid membrane of nearly 39% phosphatidylglycerol (PG) and 42% sulfoquinovosylglycerol (SQDG), and 12.5% PG and 8% SQDG, respectively. Morevoer, in experiments performed with mixtures of α- and β-CD Rawyler and Siegenthaler (1996) observed that the CD effect on the lipid depletion is additive, meaning therefore that both non-ionic and ionic lipids are extracted from the thylakoid membrane. However, Rawyler and Siegenthaler (1996) have not identified in detail the effect of the cyclodextrins on the protein components of the thylakoid membrane. In the present work, we performed a SDS-polyacrylamide gel electrophoresis experiment (see Material and Methods) to clarify further this question.

The scanning of the SDS-PAGE gel indicates that in the control thylakoid samples the molecular masses of the major polypeptides are approximately 13, 15, 17, 19, 20, 23, 28, 33, 38, 43, 48, 55, 64, 70, 80 and 125 kDa as estimated from the log MW *vs.* $\delta$ curve (see Material and Methods). The most likely assignments for these bands (in parenthesis) are: 13–15 kDa (calcium binding protein, PS II), 17, 23 and 33 kDa (oxygen evolving complex of PS II), 43 and 47 kDa (CP43 and CP47, core PS II), 19–24 kDa (LHC I complex), 24–28 kDa (LHC II complex), 55 kDa ($CF_1$ complex) and 64–120 kDa (PS I core complex with CP I and its apoproteins).

To study the effect of the cyclodextrins on the thylakoid membrane proteins, the samples were first incubated with α-, β- and γ-CD at 273 K for 10 min and then prepared for SDS-PAGE as described in the Material and Methods section. Scanning of the gels showed that there is no detectable effect of the cyclodextrins on the number and intensities of the polypetide bands, except for a slight intensity difference observed in polypeptides of 15 and 17 kDa molecular masses.

*The cyclodextrins targets on the stromal side of the thylakoid membrane*

The first question addressed here is whether the CD targets are exclusively the polar heads of the thylakoid lipids which are the galactosyl residues in DGDG, MGDG and SQDG or the phosphate group in PG (see, e.g., Murata *et al.*, 1990) as is implicitly suggested in Rawyler and Siegenthaler (1996). In this respect, the Rawyler and Siegenthaler data collected in Table I show that α-CD depletes nearly 5% MGDG, 5% DGDG, 39% PG and 42% SQDG, whereas β-CD can only deplete these lipids to a level of about 13, 15, 12% and

8%, respectively. This thereby means that α-CD is the most effective cyclodextrin for lipid depletion in the thylakoid membrane. However, this observation is in obvious contradiction with the oxygen evolution data reported in the present work where β-CD is shown to be the cyclodextrin that enhances most efficiently the activity of the oxygen evolving complex in photosystem II (cf. Table I, and Figs. 2 and 3). Thus, the data in Table I prove unambiguously that the depletion of the thylakoid membrane lipids is but one among other possible functional pathways of the cyclodextrins effect. An alternative mechanism is an effect on the thylakoid polypeptides upon incubation of the membranes with the cyclodextrins. However, the SDS-PAGE experiments discussed above indicate that in the presence of α-, β- or γ-CD no major changes in the polypeptide composition of the thylakoid membrane is apparent except for a slight loss in color intensity observed in the electrophoresis bands of the polypeptides of 15 and 17 kDa molecular masses.

The inability of the cyclodextrins to eliminate the polypeptide components of the thylakoid membrane puts the question whether the observed enhancement of oxygen evolution induced by α- and β-CD (Figs. 2 and 3) has its origin in structural changes of the photosynthetic electron transport chain polypeptides and the pigment-protein complexes. In this context, the major molecular species of the thylakoid membrane partly exposed at the stromal surface of the thylakoid membrane are the the LHCII complex, the PsaA and PsaB proteins of PSI, the CP43, CP47, D1 and D2 proteins of PSII, and the CF0-CF1 complex (see recent reports in Kühlbrandt *et al.*, 1994; Green and Kühlbrandt *et al.*, 1995; Hankamer *et al.*, 1997; Xiong *et al.*, 1998; Barber *et al.*, 1999; Klukas *et al.*, 1999; Kruse *et al.*, 2000; Zouni *et al.*, 2001). In particular, the recent models of the PSII complex published by Xiong *et al.* (1998), Barber *et al.* (1999) and Zouni *et al.* (2001) show that the molecular residues exposed at the stromal surface side are mostly located in one of the edges of the heme macrocycle in cytochrome b559, the chlorophylls in the D1 and D2 proteins, the reaction center pheophytin, and the $Q_A$ and $Q_B$ sites. In Kühlbrandt's model of the LHCII complex (Kühlbrandt *et al.*, 1994; see also Xiong *et al.*, 1998), about half of the chlorophyll molecules are exposed to the lipid environment of the thylakoid membrane which indirectly may facilitate the cyclodextrins interaction with the pigments. A reasonable conjecture is then to presume that the cyclodextrins have access to the exposed edege of the cytochrome b559 heme and/or the chlorophyll and pheophytin molecules on the stromal side of the thylakoid, thus interfering with the function of the PSII and LHCII complexes. One consequence of such physical perturbations would be a functional change in the activity of the oxygen evolving complex in PSII as the observed oxygen evolution increase in the thylakoid preparations used in this work show (cf. Figs. 2 and 3). This assumption is interesting since it is susceptible of being tested experimentally on account of the ability of the cyclodextrins to change the absorption spectra of the ligand molecule in the guest-host inclusion complex (see, e.g., Li and Purdy, 1992). To this end, the absorbance and second derivative spectra of non-treated thylakoid membranes and membranes treated with the most active cyclodextrin, i.e., β-CD, were recorded in the spectral region from 570 to 720 nm (Figs. 4 and 5).

We remark at first that the spectra of the thylakoid preparations can be resolved into five major bands visible in the absorbance and second derivative spectra at 627, 641, 650, 670, and 681 (NT) and 683 (βT) nm (cf. Figs. 4 and 5). These bands were correctly assigned in previous studies to the $Q_X$ and $Q_Y$ electronic transitions in the chlorophylls and pheophytins (cf. Table II; see also Fig. 1 in Fragata, 1991, and discussions in Fragata *et al.*, 1988; van Gurp *et al.*, 1989; Nordén *et al.,* 1992). In Fig. 4, one observes two isosbestic points, that is, identical absorbance for the same wavelength, at 580 and 675 nm in the absorption spectra of the untreated and β-CD-treated samples. The isosbestic points seen in Fig. 4 indicate that β-CD caused most likely a phase transition which might affect the stromal surface-exposed chlorophylls and pheophytins, or their environment, with a concomitant changes of their spectral properties. The presence of isosbestic points is a reliable indication, or spectral ruler, of conformational and/or geometrical changes occurring in the vicinity of the affected chromophore, meaning in the present case that the cyclodextrin-induced wavelength displacement is the result of changes in the molecular environment of the pigments.

Table II. Electronic transitions, and their respective assignments, in the red region of the spectra of thylakoid membranes before and after their incubation with β-cyclodextrin

| Type of Sample | Spectral band[a] (nm) | Electronic transitions assignments[b] | |
|---|---|---|---|
| βT[c] | 683 | $Q_Y(0,0)$ | transition in **Chl** *a* and **Phe** *a* |
| NT | 681 | $Q_Y(0,0)$ | transition in **Chl** *a* and **Phe** *a* |
| *βT, NT* | *670* | $Q_Y(0,0)$ | transition in **Chl** *a* which corresponds most likely to a monomeric form of the pigment |
| βT, NT | 650 | $Q_Y(0,0)$ | transition in **Chl** *b*; it contains also some contribution from $Q_Y(1,0)$ and $Q_Y(2,0)$ of **Chl** *a* |
| βT, NT | 641 | $Q_X(0,0)$ | transition in **Chl** *a* |
| *βT, NT* | *627* | $Q_X(1,0)$ | transition in **Chl** *a*; however, in the region from 610 to 630 nm, the $Q_X(1,0)$ transition is mixed with $Q_X(2,0)$ and $Q_Y(3,0)$ of **Chl** *a* |

[a] See Figs. 4 and 5.
[b] References: Fragata *et al.*, 1988; van Gurp *et al.*, 1989; Fragata, 1991; Nordén *et al.*, 1992.
[c] Abbreviations: Chl *a*, chlorophyll *a*; Chl *b*, chlorophyll *b*; βT, thylakoid samples treated with β-cyclodextrin; NT, control (untreated) thylakoid samples; Phe *a*, pheophytin *a*.

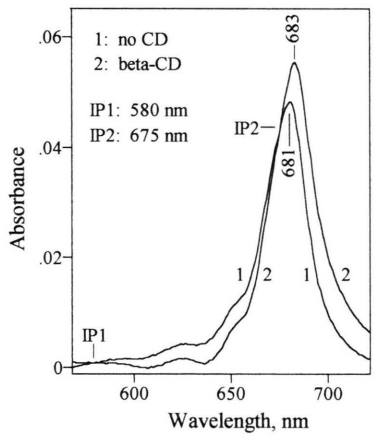


Fig. 4. Absorption spectra (570–720 nm) of control (untreated) thylakoid membranes (no CD) and membranes treated with β-cyclodextrin (β-CD). Two isosbestic points are clearly visible at 580 nm (IP1) and 675 nm (IP2). It is noted that the $Q_Y(0,0)$ transition of chlorophyll and pheophytin at 681 nm in the control (untreated) thylakoids is displaced to 683 nm in the membranes treated with β-CD. The average wavelength maxima of $Q_Y(0,0)$ in a series of six thylakoid samples are 680.9 ± 0.3 and 683.1 ± 0.2 nm.

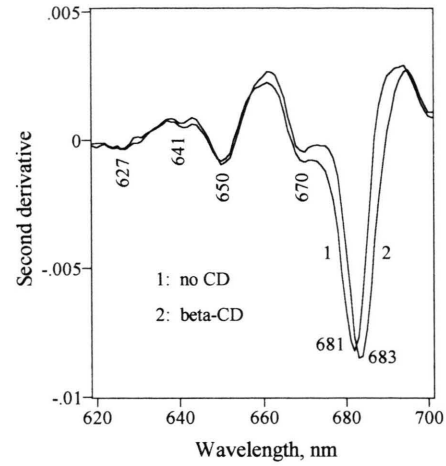


Fig. 5. Second derivative spectra (620–700 nm) of control (untreated) thylakoid membranes (no CD) and membranes treated with β-cyclodextrin (β-CD). It is noted that the $Q_Y(0,0)$ transition of chlorophyll and pheophytin at 681 nm in the control (untreated) thylakoids is displaced to 683 nm in the membranes treated with β-CD. The average wavelength maxima of $Q_Y(0,0)$ in a series of six thylakoid samples are 681.0 ± 0.2 and 683.1 ± 0.3 nm.

In Figs. 4 and 5, a significant displacement of the wavelength maximum from 681 nm (untreated thylakoid membranes) to 683 nm (thylakoid membranes treated with β-CD) is also detected. This wavelength displacement towards the red region of visible spectrum, or red-shift, is attributed to perturbations affecting the $Q_Y(0,0)$ electronic transition. In a first approximation, this red-shift is interpreted as the result of the cyclodextrins interaction with the stroma exposed edge of the pigments, i.e., a cyclodextrin-pigment interaction of the kind discussed by Li and Purdy (1992). Now, we recall that in the present work this type of interactions can only take place at the stromal surface of the thylakoid membrane as the electron microscopy experiments showed (see Materials and Methods). Since the sites of action of the CD molecules are then limited to the aqueous stroma interfaces with the lipids or the polypeptides of the thylakoid membrane, the CD-pigment interaction is therefore envisaged as the formation of a complex between the chemical groups in the wR side of the cyclodextrin (cf. Fig. 1) and the transition moment direction (μ) of the $Q_Y$ transition, $\mu_y$, or $Q_X$, $\mu_x$, in the exposed edge of the chlorophylls or the pheophytins (Fragata, 1991; Nordén *et al.*,

1992). In this context, it is important to note that in both chlorophyll *a* (Chl *a*) and pheophytin *a* (Phe *a*) the transition moment direction $\mu_y$ is at an angle near 70° with the molecular X-axis in the tetrapyrrole macrocycles of Chl *a* and Phe *a* (Fragata *et al.*, 1988; van Gurp *et al.*, 1989; Fragata, 1991; Nordén *et al.*, 1992), thereby indicating a possible CD-induced alteration of the 70° angle.

An alternative explanation of CD-induced effect on the pigment-proteins in the thylakoid membrane is to interpret the red shift of about 2 nm observed in Figs. 4 and 5, i.e., from 681 to 683 nm, as being brought about by the formation of a more compact state of the chlorophyll molecules in the LHCII or CP29 complexes. For example, close examination of the stromal surface side of the LHCII structure obtained at 3.4 Å resolution by Kühlbrandt (1994) and Kühlbrandt *et al.* (1994) shows that the nearest-neighbor chlorophylls are spaced by 9 to 13 Å (center-to-center distance). Although the dynamics of these macromolecular complexes is yet far from being known with reasonable certainty, it is predicted that any perturbation of the chlorophyll to chlorophyll distances such as those that can be induced by the CD molecules, shall cause a red-shift of the $Q_Y$ transition of chlorophyll which is exactly what the absorption and second derivative spectra in Figs. 4 and 5 show. These same arguments apply to the cyclodextrins interaction with the stromal surface side of the CP29 pigment-protein complex (see CP29 model in, e.g., Fig. 1 of Cinque *et al.*, 2000).

Finally, it is noted that the data in Figs. 2 and 3 display a good correlation with the differences in size of the inner diameter of the α-, β- and γ-CD molecules in the wide rim (wR) side (cf. Fig. 1), i.e., respectively 0.57, 0.78 and 0.95 nm (Li and Purdy, 1992). This conclusion is in agreement with previous studies illustrating the role played by the dimension of the cyclodextrins inner cavity in the formation and function of the guest-host inclusion complex in a wide variety of cyclodextrins and guest molecules (Li and Purdy, 1992; Manunza *et al.*, 1997; D'Souza and Lipkowitz, 1998; Gu *et al.*, 1999, 2000; Gu and Bayley, 2000). The foregoing interpretations are corroborated further by the data in Table I and the red-shift observed in the absorption and second derivative spectra displayed in Figs. 4 and 5. In brief, this report gives convincing evidence of the cyclodextrins usefulness as tools in the study of the stromal side of the thylakoid membrane. What is more, the application of the cyclodextrins in the investigation of the lumenal surface can as well be envisaged while using inside-out thylakoid vesicles.

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