IN SITU CROSSLINKING OF CHLOROPHYLL TO PROTEIN. USE OF SPECIFIC HETEROBIFUNCTIONAL PHOTOACTIVATED REAGENTS

by

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It is shown that reagents containing one reactive amino group and one azide group crosslink chlorophyll to protein in situ. Using the light-harvesting complex of photosystem II as model system a small fraction of the chlorophyll *a*, but no chlorophyll *b* was covalently bound to the protein. This confirms that chlorophyll *a* is more accessible from the medium. 4-Azidoaniline, 4-azido-2-nitroaniline and 2-azido-4-chloroaniline are equally effective crosslinkers and are recommended as probes for the chlorophyll organization in native or reconstituted chlorophyll-protein complexes.

1. INTRODUCTION

In higher plants light energy for photosynthesis is captured by pigments that are non-covalently bound in a number of light-harvesting protein complexes. From there it is channeled into the reaction centers. Efficient transfer of excitation energy requires that the pigments are at specific distances and angles relative to each other. To achieve this, the pigments are organized in distinct chlorophyll(Chl)-protein complexes in the thylakoids of the chloroplast.

Treatment of Chl-protein complexes with proteases has shown that practically all the Chl

is associated with the membrane-spanning parts of the polypeptides and that the pigments are not directly accessible from the water-phase (9, 20). All thylakoid Chl-proteins that have been investigated so far have a very high content of α -helices located almost vertical to the plane of the membrane, indicating that the pigments are bound between these helices (13, 14, 19, 22). Predictions of secondary structure, hydrophobicity index and accessibility of various parts of the polypeptide chain have been used to construct models of the transmembrane organization of P700 Chl_a-P1 and the light harvesting

Abbreviations: Chl = Chlorophyll; DTT = dithiothreitol; LHCII = light harvesting complex of photosystem II; PAGE = polyacrylamide gel electrophoresis; PS = photosystem; SDS = sodium dodecylsulfate; TLC = thin-layer chromatography; Tricine = N(tris(hydroxymethyl)methyl)glucine.

complex of photosystem II (LHCII) (1, 3, 4, 9, 10). It has been hypothesized that histidine or glutamine residues are involved in holding the chlorophylls in place, but we still do not know how a protein like LHCII can bind 13 Chl molecules per 25 kD polypeptide. There is no direct evidence which amino acid sidechains or which parts of the polypeptide chain are interacting with the pigments to achieve the superb degree of organization that is found in the Chl-protein complexes.

Herein, I describe a method for binding chlorophyll covalently to the polypeptides. This is a prerequisite for the determination of the environment of the pigment molecules and the identification of the chlorophyll binding sites.

2. METHODS

2.1. Biological preparations

Wild type barley seedlings (*Hordeum vulgare* cv Svalöf's Bonus) were grown for seven days in vermiculite under 16 h light (1700 lux) and 8 h dark cycles. Grana membranes were prepared as described by MØLLER and HØJ (12). The proteins were solubilized with 1% dodecyl- β ,D-maltoside and LHCII was isolated by sucrose density gradient centrifugation as described previously (8). Gradient fractions containing LHCII were quickly frozen in liquid nitrogen and stored at -30 °C. They were thawed immediately before use. Thylakoids for SDS-PAGE were prepared as described by MACHOLD et al. (11).

2.2. Synthesis of the azides from phenylene diamines

The reactions were performed under dim red light and the concentrations of the reagents were selected so that on a statistical basis only one of the two amino groups on each molecule is converted to an azide. To test the effect of substitutions on the aromatic ring on solubility and photosensitivity I prepared azido-derivatives of the following diamines: *p*-phenylene diamine, 2-nitro-*p*-phenylene diamine or 4-chloro-*o*-phenylene diamine. Suspensions (5.4 mmoles) of the diamines in 8 ml of 2 mM HCl

were stirred on dry ice to keep the temperature below -10 °C. A solution of 5.7 mmoles NaNO₂ in 3 ml H₂O was added dropwise, followed by 8 ml cold glacial acetic acid. Then 6.1 mmoles NaN₃ in 2.5 ml H₂O were added slowly. Subsequently, the mixture was diluted with 15 ml cold water and stirring was continued for 2 h at 4 °C. Then water was added to give a final volume of 100 ml and the mixture was left for 16 h at 4 °C without stirring. The reaction products - small, needle-shaped crystals - were collected by filtration. They were washed with small volume of ice-cold water and stored wet in the dark at 4 °C.

Substitutions on the ring influence the reactivity of the amino groups. Thus, one expects the following products to be formed preferentially: 4-azidoaniline from *p*-phenylene diamine; 4-azido-2-nitroaniline from 2-nitro-*p*-phenylene diamine and 2-azido-4-chloroaniline from 4-chloro-*o*-phenylene diamine.

2.3. Reaction with Chl *a*

A saturated solution of each azide was made in buffered acetonitrile (90% acetonitrile, 10% 1 M NaHCO₃ pH 9.0). An aliquot (20 μ l) of each was mixed with 25 μ g Chl *a* (Sigma) in 25 μ l acetonitrile. Oxygen was displaced by a stream of argon and the mixture was incubated under an argon atmosphere for 24 h in the dark at room temperature. As a control, pure Chl *a* was incubated under the same conditions. The reaction products were analysed by thin-layer chromatography under dim light, using pre-coated reverse-phase C-18 silica plates (Whatman) and a solvent composed of methanol:ethylacetate:water = 40:50:10 as described by HENRY et al. (7). Spots containing Chl or its derivatives were scraped off with a razor blade. The pigments were eluted with ethanol and their absorption spectra were recorded on an Aminco DW2a spectrophotometer.

2.4. Crosslinking of Chl and proteins

Thylakoid or grana membranes were resuspended in 1% dodecyl- β ,D-maltoside, 100 mM NaHCO₃ pH 9.0 to give ca 0.25 mg Chl $\cdot$ ml⁻¹ and stored on ice. The detergent was included to solubilize slowly the membranes and thereby

facilitate the interaction with the crosslinker, whilst the low temperature and the slightly alkaline buffer helped to stabilize the Chl-protein complexes. An aliquot (40 μ l) of a saturated solution of 4-azidoaniline, 4-azido-2-nitroaniline or 2-azido-4-chloroaniline in buffered acetonitrile (90% acetonitrile, 10% 1 M NaHCO_3 , pH 9.0) was added to 1 ml sample and the mixture was incubated in the dark at 4 °C for 24 or 48 h.

Alternatively, purified LHCII (0.25 mg $\text{Chl} \cdot \text{ml}^{-1}$) in 0.025% Triton X-100, 10 mM

tricine and 100 mM NaHCO_3 , pH 9.0 was incubated with 40 μ l of a saturated solution of 4-azidoaniline, 4-azido-2-nitroaniline or 2-azido-4-chloroaniline as described above. In this case no dodecyl- β ,D-maltoside was added. To activate the azide, the samples were illuminated for 10 min with strong white light in a quartz cuvette with occasional mixing. Sodium dodecyl sulfate (SDS) and dithiothreitol (DTT) were added to give final concentrations of 0.5% and 5 mM and the samples were heated in a boiling waterbath for 15 min. Aliquots containing 125

Figure 1. Separation by thin-layer chromatography of Chl *a* (lanes 1 and 5) and the derivatives formed after 24 h incubation with 4-azidoaniline (lane 2), 4-azido-2-nitroaniline (lane 3) or 2-azido-4-chloroaniline (lane 4). Panel A and B show the same plate photographed with visible light and UV light, respectively.

Aliquots were applied to a pre-coated C18 reversed phase silica thin-layer plate and developed using ethyl acetate:methanol:water = 50:40:10 as mobile phase.

Figure 2. Absorption spectra of Chl *a* and its derivatives obtained after reaction with 4-azidoaniline, 4-azido-2-nitroaniline or 2-azido-4-chloroaniline. The compounds were eluted from thin-layer plates.

μg Chl were applied for SDS-PAGE on gels containing 6 M urea (16). Covalently bound Chl was detected by its fluorescence under UV light. In some cases the fluorescent bands corresponding to LHCII or its proteolytic fragments were

excised from the urea gel, the gel slices were crushed and the protein was eluted with 5 volumes of H_2O and then with 0.05% SDS in 100 mM NaHCO_3 pH 9.0. The eluted material was lyophilized, resuspended in a small volume buffer containing 0.5% SDS, 5 mM DTT and heated again in a boiling waterbath before reapplying it to a urea gel.

3. RESULTS

3.1. Preparation of the crosslinking reagents and their reaction with Chl *a*

Chl has a keto-group on ring V that reacts readily with basic amines (21). Indeed, when Chl *a* was incubated with *p*-phenylene diamine on the bench top for 24 h in the dark and under an argon atmosphere it was converted to a product with lower mobility on reversed phase thin-layer chromatography (TLC). Therefore, azide derivatives of *p*-phenylene diamine, 2-nitro-*p*-

Figure 3. Proposed structure of the Chl *a* derivative formed with 4-azidoaniline. The formulae of the other two azidoanilines studied are also given.

phenylene diamine and 4-chloro-*o*-phenylene diamine were prepared. Adjusting the conditions so that on a statistical basis only one amino group on each molecule reacted, gave the following products: 4-azidoaniline, 4-azido-2-nitroaniline and 2-azido-4-chloroaniline. All three compounds had pronounced absorption maxima at 252 nm which decreased when the reagents were exposed to strong white light, which is typical for azides. After 10 min illumination, the absorbance at 252 nm did not decrease further. The reagents had broad adsorption maxima in the visible range plus additional minor maxima at 352 nm (4-azidoaniline) and at 361 nm (4-azido-2-nitroaniline) which disappeared completely upon illumination.

When Chl *a* (Sigma) was incubated for 24 h with the three azidoanilines in the dark and under an argon atmosphere it was converted to a form with lower mobility on reversed phase TLC (Fig. 1). After this period the reaction had almost gone to completion. The reaction products still had the pink Chl fluorescence, but the absorption maxima were shifted to 662 nm from 658 nm for unreacted Chl *a* (Fig. 2). The proposed structure of the Chl derivative after reaction with 4-azidoaniline is shown in Figure 3.

3.2. Covalent binding of Chl to protein

Initially, grana or thylakoid membranes were used for crosslinking experiments. Samples were incubated 24 or 48 h with 4-azidoaniline, 4-azido-2-nitroaniline or 2-azido-4-chloroaniline in the dark before activation of the azido-groups by strong white light. Then the samples were analysed by SDS-PAGE under denaturing conditions (Fig. 4A and C). In control samples, all the Chl migrated with the free pigment at the front. In crosslinked samples LHCII was most strongly labelled with Chl, but there were also faint bands at the position of other Chl proteins from photosystem II. In addition, there was a fluorescent band below LHCII, where normally no green band is observed. This may be due to proteolytic breakdown products.

To simplify the interpretation of the gel pattern and because it was the most strongly labelled band, isolated LHCII was chosen as a

model system. The outcome of one crosslinking experiment is shown in Figure 4B. Most of the Chl still migrated with the free pigment, suggesting that not all Chl molecules were equally accessible to the crosslinker. When the band corresponding to LHCII was excised and the material was applied to electrophoresis in a second urea containing gel, all the pigment comigrated with LHCII polypeptides and there was no detectable free Chl (Fig. 4D).

Absorption spectra of LHCII eluted from SDS polyacrylamide gels after crosslinking exhibited a single absorption maximum in the red at 662 nm, the same wavelength as the Chl *a* derivatives, suggesting that only Chl *a* had reacted with the crosslinker (Fig. 5). This is in agreement with previous findings indicating that Chl *a* is much more accessible from the medium than Chl *b*. It was not possible to quantitate the amounts of Chl that was covalently bound to the protein, but clearly only a small fraction of the Chl molecules had reacted, either due to steric hindrance or because only a small subset of peripherally located Chl *a* was accessible to the reagents.

4. DISCUSSION

The identification of the Chl-binding sites of Chl-proteins from higher plants is seriously hindered by the fact that the pigments are bound to the proteins by non-covalent interactions. Crystals have been obtained of reaction center complexes from several photosynthetic bacteria, and their three-dimensional structure has been determined so that the orientation of the reaction center pigments is known, but it is still not certain with which amino acid residues they interact (2, 5). For thylakoid proteins from higher plants, there are so far no satisfactory three-dimensional crystals available. Therefore, it is proposed to analyse the environment and the organization of the Chl molecules in thylakoid proteins by crosslinking the Chl to the protein, digesting the polypeptide chain with proteases and determining the amino acid sequence of the fragments labelled with Chl.

A good crosslinking reagent has to fulfil several conditions. It should react specifically with

Chl, but the reaction with the polypeptide chain should be as unspecific as possible. This condition is met by azide derivatives of aromatic diamines. Chl has a keto-group on ring V that reacts with basic amines (21). The reaction proceeds at low temperature and at pH values where Chl-proteins are not denatured. Considering that the Chl is not directly accessible from the water phase, the reagents should be amphiphilic, i.e. they should be somewhat water soluble, but lipophilic enough to diffuse into the thylakoid membrane, and they should be small, to be able to get into Chl-binding pockets or folds to reach Chl that is bound between subunits of Chl-protein complexes. Based on these considerations azido-derivatives of the following compounds were prepared: *p*-phenylene diamine, 2-nitro-*p*-phenylene diamine or 4-chloro-*o*-phenylene diamine. The products 4-azidoaniline, 4-azido-2-nitroaniline and 2-azido-4-chloroaniline reacted readily with Chl *a* in the dark, giving rise to products with lower mobility on reversed phase TLC. The reaction products still had the pink Chl fluorescence, but their absorption maxima were shifted by 4 nm to the red.

When thylakoid or grana membranes were treated with these crosslinkers, only a small part of the Chl could be covalently bound to the

Figure 5. Absorption spectra of LHCII with covalently bound Chl isolated by SDS-PAGE on gels containing 6 M urea.

proteins. LHCII was the most prominently labelled band, but Chl fluorescence was also detectable at the position of other Chl-proteins. The differential labelling may reflect the relative abundance of the Chl-proteins. About 80% of the Chl present in grana membranes is associated with LHCII. The amount of Chl bound did not change when the incubation time was varied between 24 and 48 h, suggesting that the limiting

Figure 4. Denaturing SDS-PAGE of solubilized thylakoid membranes and purified LHCII after crosslinking with azidoanilines. Aliquots containing ca 125 µg Chl were heated in a boiling waterbath before application to gels containing 6 M urea. The gels were photographed under UV light.

A: Crosslinking of thylakoid membranes using 2-azido-4-chloroaniline (lane 1), 4-azido-2-nitroaniline (lane 2) or 4-azidoaniline (lane 3). Lane 4: control without crosslinker.

B: Crosslinking of purified LHCII. The samples labelled (+) were exposed to 25 µg protease V8 for 90 min at room temperature.

Lane 1) Control

Lane 2) 4-azido-2-nitroaniline

Lane 3) 2-azido-4-chloroaniline

C: Another crosslinking experiment using thylakoid membranes.

Lane 1) 4-azidoaniline

Lane 2) 4-azido-2-nitroaniline

Lane 3) 2-azido-4-chloroaniline

Lane 4) control

D: After crosslinking the LHCII band was excised from an SDS-urea gel, the protein was eluted and reapplied to SDS-PAGE on a gel containing 6 M urea.

Lane 1) Grana membranes crosslinked with 4-azidoaniline

Lanes 2-4) LHCII after crosslinking with 4-azidoaniline (2), 4-azido-2-nitroaniline (3) or 2-azido-4-chloroaniline (4) isolated by denaturing SDS-PAGE.

factor was the accessibility of the pigments rather than the formation of the Schiff base. Similar crosslinking yields were obtained using the three differently substituted azidoanilines. This may be due to excitation transfer to the crosslinker from neighbouring Chl molecules. It has been shown that Chl and other porphyrins can be used to photosensitize reactions (6).

Experiments with isolated LHCII revealed that only Chl *a* was bound by the crosslinker, thus confirming that Chl *a* is much more accessible from the water phase than Chl *b*. This result, in combination with the low crosslinking yield, indicates that only the outermost layer of Chl was accessible to the crosslinker.

The crosslinking method presented here seems suitable to identify Chl binding sites of Chl-proteins with known amino acid sequence. At the same time the accessibility of Chl molecules can be explored and yield information about the three-dimensional structure of Chl-protein complexes. Since it is possible to reconstitute LHCII with pigments (17) a further approach is to react isolated Chl *a* and *b* with the crosslinker in the dark, reconstitute the pigments with the protein and then crosslink the Chl to the protein. This is expected to give much higher crosslinking yields and permit the identification of Chl-binding domains that are not accessible from the outside.

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