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Interaction of chlorophyll *a'* with the 65 kDa subunit protein of photosystem I reaction center

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The 65 kDa polypeptide subunit depleted of P700 was prepared from a photosystem I reaction center preparation and mixed with chlorophyll *a'* (C-10 epimer of chlorophyll *a*) to yield a complex exhibiting a triple-headed spectrum with absorbance maxima at 673, 692 and 707 nm. The difference spectra (oxidized-minus-untreated and light-minus-dark) had a major trough at 707 nm and minor ones at 690 and 430 nm. The overall shape of the spectra resembled well that of P700 with a small red shift. A rapidly decaying flash-induced absorbance change was observed at 430 nm with a half decay time of less than 500 μs in a preparation supplemented with an electron donor system.

Chlorophyll *a'*; P700; Photosystem I; Photosynthesis

1. INTRODUCTION

It has been well established through numerous works since its discovery by Kok [1] that P700 is the reaction center pigment, i.e. the primary photochemical electron donor of photosystem I. The chemical nature of P700 has not been established: dimeric chlorophyll *a* (Chl *a*) bound to a protein [2], a Chl *a* enol [3], and a chlorinated and hydroxylated derivative [4] have been proposed. Our high-performance liquid chromatography (HPLC) analysis [5] revealed that intact leaves of various plants contained a small amount of Chl *a'* (C-10 epimer of Chl *a*). In [6] the Chl *a'*/P700 ratio was determined to be about 2, but a more recent work with improved HPLC resolution gave a value closer to unity [7]. This at least suggests that Chl *a'* is an integral part of P700. A recent work has indicated that the essential part of the photosystem I reaction center assembly might be quite simple. The large subunit(s), most likely a complex of two, is known to contain P700 as well as the primary electron acceptors [8]. Here, we have isolated a P700-free preparation of the large subunit(s), tentatively called the 65 kDa protein, and investigated the interaction between Chl *a'* and this apoprotein.

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2. MATERIALS AND METHODS

The P700-free 65 kDa protein was prepared as follows: a Triton-solubilized photosystem I pigment-protein complex prepared as in [8] was incubated under N_2 in a medium containing 1% lithium dodecyl sulfate (LDS, purchased from Sigma), 50 mM Tris-HCl, pH 8.8, and 20% sucrose for 1 h at 0°C in the dark. 1 ml of the LDS-treated preparation was loaded on a Biogel P-200 column (2.6×90 cm) equilibrated with 0.05% LDS, 50 mM lithium citrate, pH 8.0, and 10% sucrose. The medium was flushed with nitrogen several times before use. The column was then eluted with the above medium. Only one major peak was detected as monitored at 280 nm, which

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was followed by visually green but much less 280-nm-absorbing fractions much later. These major peak fractions were collected and concentrated by using an ultrafiltration membrane (UP-20, Toyo Roshi). The concentrated preparation (protein concentration, ~1 mg/ml, rough estimate from absorbance at 280 nm) appeared slightly green (cf. spectrum in fig.1). SDS-polyacrylamide gel electrophoresis (SDS-PAGE) revealed only one peptide band at 65 kDa.

Chl *a* (99.9%) and Chl *a'* (with 0.5–1% Chl *a*) were prepared as in [9]. Bovine serum albumin (BSA) was from Sigma (catalog no. A4378). N_2 gas with less than 0.5 ppm O_2 was used without further treatment. Absorbance spectra, both absolute and difference, were measured by using a spectrophotometer (model 557, Hitachi) connected to a personal computer (PC8801, NEC). Flash spectrophotometry was performed basically as in [10] except for a longer flash pulse width (100 μs).

A typical reconstitution experiment was performed as follows: 1 ml protein solution (~1 mg/ml) in 50 mM Tris-HCl, pH 8.8, 20% sucrose, and 0.05% LDS was flushed several times with N_2 in a rubber-stoppered tube. Crystalline Chl *a'* was dissolved in ethanol which had also been flushed with N_2 (final concentration, 250 μM). Then, 20 μl of this ethanol solution was injected through the stopper using a microsyringe into the protein solution. In most of the experiments, the tube was placed in the cuvette holder of the spectrophotometer with the aid of an adaptor. For difference spectra, the solutions were transferred to an open micro-cuvette (light path, 10 mm) without special precautions to make the system anaerobic.

3. RESULTS

The 65 kDa protein preparation separated by LDS gel-permeation chromatography (see section 2) lacked P700 activity, either photooxidation or chemical oxidation-reduction. A small amount of chlorophyll (<0.4% of the original) remained bound. Immediately after addition of Chl *a'* (~1/3 of the number of protein molecules) two distinct absorbance peaks at 692 and 707 nm appeared besides a larger peak at 673 nm (fig.1, broken line). Within the next 30 min, the 692 and 707 nm peaks grew larger at the expense of the 673 nm peak. The steady state seemed to have been

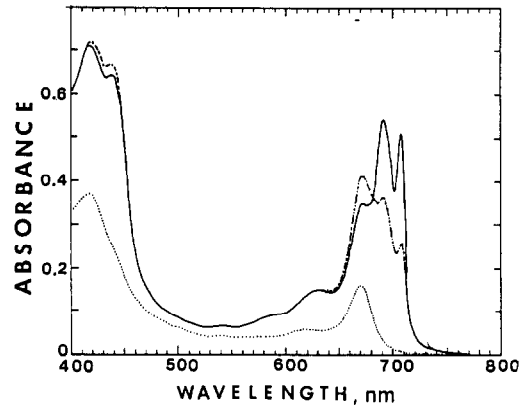

Fig.1. Spectra of Chl *a'*-supplemented 65 kDa protein preparations. (···) Absorbance spectrum of the 65 kDa apoprotein before the addition; (— — —) immediately after addition of Chl *a'* ethanol solution; (——) 40 min after the addition. See text for further details.

attained in 40 min at room temperatures (fig.1, solid line). This triple-headed spectrum is quite similar to that previously reported for dimeric Chl *a'* in aqueous methanol [6].

Addition of 0.1 mM potassium ferricyanide led to a 10% decrease in the 707 and 692 nm peaks. On the other hand, a reductant system, ascorbate and *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD), caused no effect at all. This indicates that the newly formed Chl *a'*-protein complex was in its reduced form. Illumination of the complex with intense white light also induced a spectral change similar to that caused by chemical oxidation though somewhat smaller. These difference spectra are shown in fig.2. For comparison, the flash-induced light-minus-dark difference spectrum of P700 in a digitonin-treated spinach preparation is shown in the upper part of fig.2. The major peak in the Chl *a'* complex is at 707 nm with a subpeak at 690 nm, while that for P700 was at 703 nm with the subpeak at 683 nm.

The oxidation described above, either chemical or light-induced, was virtually irreversible. The addition of excess ascorbate and TMPD or phenazine methosulfate to either ferricyanide-oxidized or illuminated preparations did not significantly change the spectrum back to the original. The absorbance changes at 430 nm induced by red flash (100 μs) excitation seemed to be at least partly reversible (fig.3). The change induced by the se-

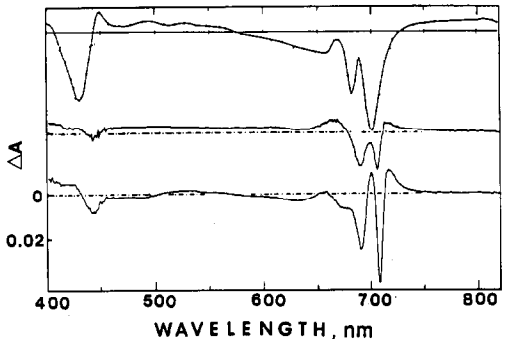

Fig.2. Difference spectra of P700 and the Chl *a'*-supplemented protein. (Upper trace) Light-minus-dark difference spectrum of P700 adapted from the original data in [11]; (middle trace) light-minus-dark difference spectrum of Chl *a'*-protein complex; (lower trace) oxidized-minus-untreated difference spectrum of Chl *a'*-protein complex. Oxidation was performed by adding potassium ferricyanide (0.1 mM).

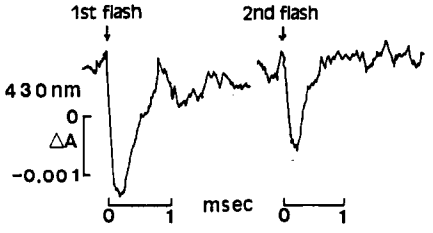

Fig.3. Flash-induced absorbance changes of the Chl *a'*-protein complex (see text for details). Reaction mixture contained 1 mM sodium ascorbate, 0.1 mM TMPD, and 50 mM Tris-HCl (pH 8.8) at room temperature.

cond flash was significantly smaller than that of the first. A prolonged dark period between the two flashes did not improve this situation, and after several flashes the change became too small to be separated from the noise. This type of partially reversible change took place only in a preparation that had been supplemented with TMPD-ascorbate prior to addition of Chl *a'*. Addition of TMPD-ascorbate after reconstitution was ineffective.

The characteristic triple-headed spectrum shown above was not obtained when Chl *a* was used in place of Chl *a'* (fig.4, dotted line). A small shoulder around 700 nm did not decrease upon addition of ferricyanide, indicating that this component is different from the 707 nm component of the Chl *a'* complex. Subsequent addition of Chl *a'*

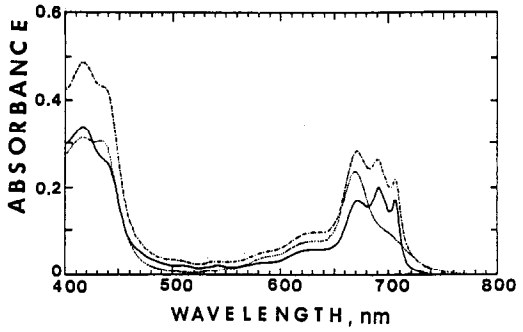

Fig.4. Spectra of Chl *a*- and Chl *a'*-supplemented 65 kDa protein preparations. Chl *a'* alone (——), Chl *a* alone (···); Chl *a'* and Chl *a* (– – –).

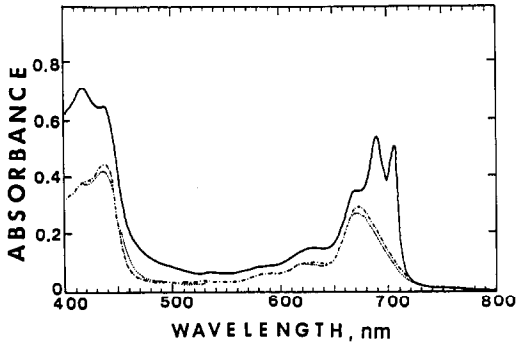

Fig.5. Spectra of Chl *a'*-supplemented preparations. Chl *a'* added to the 65 kDa protein (——); added to the 0.05% LDS medium (– – –); added to BSA (1 mg/ml) in the LDS medium (···).

to this preparation induced a triple-headed spectrum (broken line) semilar to that with Chl *a'* alone (solid line). As reported in [6], Chl *a* alone in aqueous methanol exhibits a broad, single peak.

To rule out the possibility that any other protein or even LDS alone, a detergent present in the medium, might be sufficient to provide a favorable environment for formation of the complex, the specificity of the 65 kDa apoprotein was investigated using BSA and LDS in place of the 65 kDa protein. As shown in fig.5, neither BSA nor LDS was capable of inducing a characteristic triple-headed spectrum.

4. DISCUSSION

The present results indicate that Chl *a'* forms a complex with the 65 kDa protein, one of the photosystem I reaction center subunits. This Chl

a'-protein complex with its characteristic triple-headed absorbance spectrum undergoes an oxidation-reduction cycle similar to that of P700, either chemically or photochemically, although its reversibility is limited. The difference spectra are perhaps much closer to that of P700 than any other in vitro models of P700 that have thus far been claimed. Our previously reported in vitro preparation, i.e. Chl *a'* in aqueous methanol [6], was also closer at that time than any other previously described systems with Chl *a* and aqueous organic solvents in terms of the difference spectrum. The fact that the difference spectra of the present complex with Chl *a'* and the P700 apoprotein are even closer to that of P700 indicates that Chl *a'* is essential for the formation of P700, and that this specific protein provides an environment more favorable than aqueous organic solvents. A recent analysis [7] revealed that the ratio of Chl *a'* to P700 was closer to unity than the previously reported value of 2 [6]. This suggests three possible compositions of the reaction center pigments: (i) One molecule of Chl *a'* plus other pigment(s), most likely Chl *a*; (ii) One molecule of Chl *a'* alone; (iii) Two molecules of Chl *a* plus one molecule of Chl *a'*. In the present experiments, the spectral properties reminiscent of P700 were generated exclusively by Chl *a'*, not by Chl *a*. However, this does not necessarily rule out the first one because our apoprotein preparation contained some Chl *a* which were firmly bound despite harsh detergent treatment as observed in fig.1 (dotted line). There is even another possibility still open that the core of P700 is a dimer of Chl *a'*, because the estimation of P700 was based on an extinction coefficient obtained in a digitonin-treated preparation and the ratio thus obtained may not be absolute and be subject to fluctuation.

It should be noted that, althoug our Chl *a'*-protein complex exhibited a redox difference spectrum fairly similar to that established for P700, there are still some discrepancies to be clarified between the two entities. One is the virtual irreversibility of oxidation, whether chemical or photochemical. This may be due to the lack of primary and secondary electron acceptors which could well have been removed during the preparation procedure. Another is the imperfect coincidence of the difference spectra between the complex and that of P700. Partial denaturation and inactivation of the apoprotein could well be responsible as well as the loss of electron acceptors. It is also possible that Chl RCI is needed to complete the reconstitution, as the Chl *a'*/Chl RCI ratio was found to be unity in some photosystem I particles (Senger, H., personal communication).

With the present experiment as the first step, a more systematic and quantitative study is currently underway. This includes an improvement on the preparation procedures to obtain less denatured apoprotein and characterization of the Chl *a'*-protein complex with more structure-sensitive means such as CD and ESR.

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