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A NON-FLUORESCENT COMPLEX OF CHLOROPHYLL a WITH PLASTOCYANIN

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SUMMARY: A complex between chlorophyll a and plastocyanin has been prepared by dialysis of mixtures of chlorophyll in Triton X-100 micellar solution with the protein. The complex appears to contain no more than one chlorophyll per plastocyanin molecule, and is non-fluorescent, whether the protein is in the oxidized or reduced state. The lack of fluorescence suggests that the chlorophyll is adsorbed very close to the Cu center.

It has by this time been quite well established that Cu (I) plastocyanin is the primary electron donor to oxidized Photosystem I, $P700^+$, in higher plants. Its role is apparently to transfer electrons from the cytochrome b_6-f complex to oxidized chlorophyll. In order to accomplish this transfer, plastocyanin binds alternately to the cytochrome b_6-f complex (1) and to Subunit III of Photosystem I particles (2,3). The presence of cations is known to facilitate proper docking of plastocyanin at its PS I binding site (2,4,5), but it would not be surprising if hydrophobic binding and recognition of chlorophyll in PS I were important too.

Although complexes between chlorophyll and several proteins have been prepared *in vitro*, none, to our knowledge, has hitherto been prepared with plastocyanin, in spite of its obvious biological relevance. We report the preparation of an apparently 1:1 complex between chlorophyll a and plastocyanin, and that it is non-fluorescent in either the Cu (II) or Cu (I) oxidation state.

MATERIALS AND METHODS

Plastocyanin was prepared from spinach acetone powder by the procedure of Katoh et al. (6) and chromatographed three times on Whatman DE52 diethylamino-

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ethyl cellulose columns, the last time with a 0.030 - 0.075 M Na phosphate buffer (pH 7.8) gradient. The sample used had a 275 nm/595 nm absorbance ratio of 1.48, and a plastocyanin concentration of 1.35×10^{-4} M, in phosphate buffer.

A stock solution of chlorophyll in Triton X-100 was prepared as follows. Chlorophyll in ether was evaporated, redissolved in 0.100 ml of 95% ethanol, treated with 0.150 ml of Triton X-100, 0.104 M in water, and diluted to 5 ml with 0.050 M phosphate buffer, pH 7.8. The resulting Triton concentration was 3.12×10^{-3} M, and the chlorophyll concentration was 8.0×10^{-5} M, assuming a molar absorptivity of 63000 at 669 nm (7). If a critical micelle concentration (cmc) for Triton X-100 of 2.5×10^{-4} M is assumed (8), and an aggregation number of 43 (9), the ratio of micelle to chlorophyll concentration was approximately 0.83.

Formation of the complex.- Solutions of plastocyanin and of chlorophyll-Triton were mixed, and dialyzed for 24 h against a 100-fold excess of .050 M phosphate buffer. This procedure lowered the Triton concentration inside the bag to well below the cmc, forcing chlorophyll either to aggregate, adsorb to the dialysis membrane, or bind to the protein. Recording the absorption spectra on a Cary 14 spectrophotometer before and after dialysis afforded information as to the fate of the chlorophyll (and the plastocyanin).

RESULTS

The formation of complex from 1 ml of chlorophyll-Triton solution and 2 ml of plastocyanin solution is shown in Fig. 1. The chlorophyll band, in the spectrum of the inner phase after dialysis, remains near 669 nm but appears

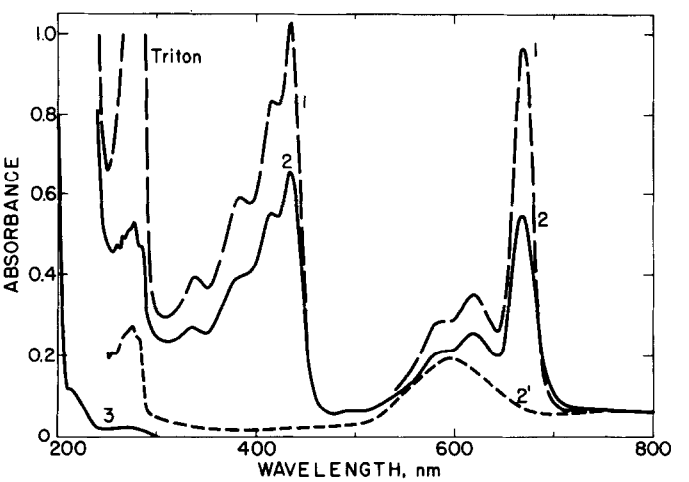

Fig. 1. Preparation and dialysis of a complex between chlorophyll a and plastocyanin. Trace 1: Spectrum of the solution of plastocyanin mixed with chlorophyll in Triton X-100 before dialysis. The Triton absorption band in the ultraviolet is indicated. 2: Spectrum of the inner phase after dialysis against 50 mM phosphate buffer. 2': The estimated contribution of plastocyanin to the spectrum of Trace 2. 3: Spectrum of the outer phase after dialysis, showing only the 100-fold reduced bands of Triton.

somewhat broadened. About half the chlorophyll is lost, probably adsorbed to the dialysis tubing. From the spectrum, the concentration of chlorophyll in this inner phase is about 1.6 X 10^{-5} M, and that of plastocyanin about 8 X 10^{-5} M, five times as large. The spectrum of the outer phase shows only the much reduced absorption of Triton. The result was similar when a preparation was made with half as much chlorophyll initially, as only half as much chlorophyll was found in the inner phase with plastocyanin. When the complex was prepared with chlorophyll in excess (3.2 X 10^{-7} mol compared to 1.35 X 10^{-7} mol plastocyanin), about half of the chlorophyll in the inner phase was found in an aggregated form with an absorption band at 746 nm. This form is probably not associated with protein. The amount of chlorophyll in the 669 nm form was estimated to be 75-80% of the amount of plastocyanin present.

It was conceivable that the 669 nm, apparently monomeric, form of chlorophyll was present in solution with plastocyanin but was not bound to it. However, this chlorophyll migrated down a Sephadex G10 column along with the plastocyanin, without holdup, and was chromatographed on a Sephadex G25-80 column in the same fraction as the plastocyanin. The 746 nm aggregate, however, remained at the top of the latter column and would not move until a Triton solution was applied. We conclude, therefore, that 669 nm chlorophyll is bound to the plastocyanin.

Usually, when chlorophyll or one of its derivatives is bound to proteins in a monomeric form, its solutions are fluorescent (10-15). However, although chlorophyll in micellar Triton solutions is visibly fluorescent, no fluorescence whatsoever could be seen in solutions with plastocyanin under "black light" irradiation, or when recorded on our spectrofluorometer (16). An upper limit of about 0.003 could be set to the quantum yield of fluorescence from the spectrofluorometer trace, but no fluorescence band could in fact be discerned. Nor could fluorescence be elicited after reduction of plastocyanin with ascorbate. This lack of fluorescence from a sample of apparently monomeric chlorophyll is most unusual.

DISCUSSION

The structure of plastocyanin shows Cu to be bound close to the surface at one end of the molecule, by two His, one Cys and one Met residues (17). The Cu is surrounded by a "hydrophobic patch" of seven amino acids. There is also an elongated negatively charged region along one side of the molecule, which is probably involved in binding to P700 through cations (18,19). Binding of redox agents at either of these sites, but especially at the hydrophobic patch, leads to electron transfer reactions with plastocyanin (20-22).

Experimentally, we have found no evidence for the association of more than one molecule of chlorophyll with one molecule of plastocyanin. This follows from the invariance of the chlorophyll absorption band shape with the ratio of chlorophyll to protein, and from the formation of the 746 nm microcrystalline hydrate species when chlorophyll is in excess. This, together with the lack of fluorescence, suggest that the bound chlorophyll is located very close to the Cu. Perhaps the chlorophyll is attracted to the hydrophobic patch, and perhaps it is ligated to the more exposed His 87 residue around the Cu. It also cannot be excluded that one or more Triton molecules remain associated with the chlorophyll-protein complex.

Fluorescence may be quenched by energy or electron transfer, or less likely by Cu-induced intersystem crossing between chlorophyll excited states. In Cu (II) plastocyanin, the most obvious process is energy transfer to the Cys-Cu charge transfer bands near 13000 cm^{-1} (23), though electron transfer (reduction of Cu (II)) is also a possibility. Cu (I) plastocyanin has no absorption bands suitably located for energy transfer, and the failure to restore fluorescence on reduction by ascorbate leaves electron transfer (oxidation of Cu (I)) as the only obvious mechanism, and one which is certainly permitted energetically. If that is so, the charge-transfer transient must be very short-lived, as we could not detect photoreduction of triphenyltetrazolium chloride added to the solution in the presence of excess ascorbate. It is interesting to recall that Hirsch and Brody reported a

photochemical reaction involving chlorophyll and oxidized plastocyanin in mixed monolayers (24).

The evidence that chlorophyll binds very close to the Cu of plastocyanin *in vitro* raises the possibility that recognition of chlorophyll in P700 by the hydrophobic patch of the protein is part of the process of docking to P700 proteins, along with ionic binding mediated by cations.

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