TRACE METAL COMPOSITION OF FRACTIONS OBTAINED BY DIGITONIN FRAGMENTATION OF SPINACH CHLOROPLASTS

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Received September 24, 1964

According to current concepts the process of photosynthesis in green plants involves two distinct primary photochemical reactions (cf. review by Clayton, 1963). These two reactions are thought to be mediated by the light absorbed by different pigment systems, which have been denoted pigment system 1 and pigment system 2 (Duysens, 1963). Absorption of light by system 1 results in the reduction of NADP and the oxidation of a cytochrome, while light absorbed by system 2 causes reduction of the cytochrome and oxidation of water with resultant oxygen evolution. Thus the products of the light phase of photosynthesis, $NADPH_2$, ATP and oxygen result from the co-operation of the two systems.

By fractionating the chlorophyll-protein complexes obtained after incubation of spinach chloroplasts with digitonin we have recently obtained evidence that the chlorophyll of spinach chloroplasts is associated with two different types of particles (Boardman and Anderson, 1964). One type of particle is more soluble in digitonin and has a chlorophyll a/b ratio of 5.0 to 6.0; whereas the other type of particle remains more strongly attached to the structural proteins of the grana and has a lower chlorophyll a/b ratio (2.0). For intact chloroplasts this ratio was

3.0. Since the particles with the high chlorophyll a/b ratio are able to photoreduce NADP only in the presence of the electron donor couple (ascorbate and indophenol dye), it was speculated that these particles contain pigment system 1, while the particles less soluble in digitonin may represent an integral part of system 2. This present communication provides further evidence that these two fractions correspond to systems 1 and 2. This evidence is based on the distribution of Cu, Mn and Fe in the two fractions.

Spinach chloroplasts were prepared by blending leaves in phosphate buffer (0.05M, pH 7.2) containing 0.3M sucrose and 0.01M KCl and sedimenting the filtered homogenate at 1000 x g for 10 min. The phosphate buffer was rigorously purified by extraction with 8-hydroxyquinoline in chloroform to remove traces of metals (Gentry and Sherrington, 1950). For digitonin fragmentation, spinach chloroplasts were suspended in purified phosphate buffer (0.05M, pH 7.2) containing 0.01M KCl and 0.5% digitonin (twice recrystalised in ethanol) and incubated for 30 min at $0^{o}C$. The preparation was then separated by differential centrifugation in the following manner; 10 min at 1000 x g, 30 min at 10 000 x g, 30 min at 50 000 x g, and 60 min at 144 000 x g. Prior to chemical analysis, all samples (whole chloroplasts, the 10 000 x g and 144 000 x g fractions) were washed twice by resuspension in glass-distilled water and recentrifugation at the appropriate speeds, and then lyophilized.

For determination of the metal components, the lyophilized fractions (20-50 mg) were digested with 2 ml of H_2SO_4 - $HClO_4$ (1+7) and 5 ml of conc. HNO_3. After digestion, 10 ml of water was pipetted into flasks, the

contents thoroughly mixed and filtered through dry Whatman No. 42 papers (9 cm). Copper, iron, manganese and magnesium were determined on these filtrates or dilutions of them, against standards containing in parts per million : Cu, 0-0.5; Fe, 0-6; Mn, 0-3; and Mg, 0-1 each at suitable concentration intervals and H_2SO_4 at a concentration equivalent to sample solutions. A Perkin Elmer Model 303 atomic absorption spectrophotometer was used with the following instrument settings:

Hollow cathode tube current: 18 ma for Cu, 40 ma for Fe, 20 ma for Mn, 15 ma for Mg.

Slit width: 1.0 mm for Cu, Mn and Mg; 0.3 mm for Fe.

Flame: Stoichiometric air-acetylene mixture, 10 cm path-length.

Resonance lines: Cu 3247 Å, Fe 2483 Å, Mn 2795 Å, Mg 2852 Å.

Scale expansion: 2-fold for Fe and Mn, 5-fold for Cu, unexpanded for Mg.

The results obtained are shown in Table I. The most striking partitioning was in the case of manganese. The 10 000 x g fraction which we believe to be more representative of a system 2 (i.e. O_2-evolving) than system 1 particle contained 4.5 times as much manganese (relative to the magnesium content) as the 144 000 x g fraction (representative of a system 1 particle). From other evidence, manganese is believed to be required for the oxygen-evolving sequence of photosynthesis (Kessler, 1957; Spencer and Possingham, 1961). On the other hand the copper and iron contents of the 144 000 x g fraction were higher than in the 10 000 x g fraction. Iron is of course implicated since the cytochromes are involved in photosynthetic electron transport (Duysens, 1963). Copper is

TABLE I

Trace Metal Content of Chloroplast Fractions

The Mn, Fe and Cu contents of the fractions are given relative to Mg content. Magnesium was shown to be nearly 100% equivalent to the chlorophyll content of these fractions. The magnesium content in umoles/g dry wt. was 90.4 for chloroplasts, 102.4 for 10 000 x g fraction and 62.5 for 144 000 x g fraction respectively.

Fraction	Mg	Mn	Fe	Cu	Chlorophyll a/b
Chloroplasts	100	1.37	4.16	1.88	3.0
10 000 x g	100	1.93	2.11	1.00	2.0
144 000 x g	100	0.41	3.41	1.62	5.5
Quantosomes[*]	115	1	6	3	2.3[≠]

* Park & Pon (1963) ≠ Lichtenthaler & Calvin (1964)

also known to be present since a copper-containing protein, plastocyanin has been located in chloroplasts by Katoh et al. (1962). Recent works by Katoh (unpublished) and Smillie (unpublished) indicate that plastocyanin is located on the system 1 side of the electron transport pathway. It is therefore of interest that the iron and copper contents of the 144 000 x g fraction are higher than in the 10 000 x g fraction. Comparison of the metal content of these fractions with the quantosomes of Park and Pon (1963) show that their values are closer to those of intact chloroplasts, although they are somewhat higher.

It is worthwhile to note that the likelihood of adsorption of heavy metal ions to chloroplasts and chloroplast fragments is very great, and therefore prior purification of buffers used for isolation is essential. A similar

experiment to that shown in Table I, in which the three fractions were isolated with unpurified phosphate buffer, resulted in higher values for the trace metals in all fractions; the iron content was particularly high.

This data shows that digitonin fragmentation of spinach chloroplasts for 30 min. results in chlorophyll-containing particles not only with different amounts of chlorophyll a and b and different photochemical activities, but also with different ratios of trace metals. Moreover the distribution of these metals between the two chloroplast fractions is consistent with our hypothesis that we have obtained a physical separation of system 1 particles from particles which are more representative of system 2 than system 1. Analysis of the cytochrome content of these fractions will be of great interest. Further work is in progress to determine the structure, composition and function of these chlorophyll-containing particles and full details will be published elsewhere.

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