Plant Physiol. (1977) 59, 1178-1181

Strong Binding of Cytochrome *c* on the Envelope of Spinach Chloroplasts

Received for publication November 10, 1976 and in revised form December 16, 1976

MICHEL NEUBURGER, JACQUES JOYARD, AND ROLAND DOUCE
Laboratoire de Biologie Végétale, Département de Recherche Fondamentale, Centre d'Etudes Nucléaires et Université Scientifique et Médicale de Grenoble, 85 X 38041 Grenoble, France

ABSTRACT

Yeast cationic ferricytochrome *c* was able to bind to the spinach (*Spinacia oleracea*) chloroplast envelope with a low affinity (K*d* = 1.1 μM). The total amount of low affinity binding sites was of the order of 50 nmol cytochrome *c* mg^{-1} protein. We gave the evidence that binding of ferricytochrome *c* to the envelope was electrostatic and that the envelope membranes were strongly negatively charged. Addition of yeast ferricytochrome *c* to a preparation of intact washed chloroplasts (class I) induced a strong agglutination of chloroplasts.

Very recently, Billecocq (2) has shown that the anionic polar head (sulfoquinovosyl pole) of the sulfolipid in the envelope is accessible to the specific antibody and directed toward the outside. Recent studies (1, 28) have led to the proposal that the isoelectric point of class I chloroplasts determined by cross-partition is acidic. Hence, at physiological pH, the surface of the envelope is probably negatively charged. Polycationic Cyt *c* (5) was used as a probe to determine the importance and the exact nature of the charges on the surface of the envelope membranes.

MATERIALS AND METHODS

Chloroplast Preparation. Intact spinach (*Spinacia oleracea*) chloroplasts capable of high rate of CO_2 fixation were prepared from expanding spinach leaves (approximately 8 weeks old) according to the method of Heber (10). The percentage of intact chloroplasts in a preparation was determined by phase microscopy (16) and by its ability to evolve O_2 in the presence of ferricyanide before and after an osmotic shock (16). Envelope membranes and thylakoids were isolated according to previous methods (6, 13, 15) and resuspended in the following medium: 0.3 M sorbitol and 1 mM Tricine-NaOH buffer (pH 7.2). For some experiments and in order to eliminate excess Mg, intact chloroplasts were washed in the following medium: 0.3 M sorbitol, 1 mM Tricine-NaOH buffer (pH 7.2), 2 mM EDTA, and spun at 2,000*g* for 2 min (washed chloroplasts.)

Preparation of ^{59}Fe-Cytochrome *c*. Yeast cells (D 261 strain) were kindly given by Prof. P. Vignais. The glycerol culture medium (24) contained $^{59}FeCl_3$ (0.3 mCi l^{-1}). For isolation of yeast ^{59}Fe-Cyt *c*, 0.5 kg of labeled yeast was suspended in 20 mM phosphate buffer (pH 8.5) containing 1.2 M NaCl and broken at 5 C in a french press (40,000 p.s.i.). The homogenate was then centrifuged for 20 min at 20,000*g*. The yellow-red supernatant containing the Cyt *c* was diluted 10-fold and added to 8 g of CM-Sephadex C-50 (Pharmacia) previously equilibrated with 0.1 M phosphate buffer (pH 7.2). After shaking for 30 min, the resin containing the bound Cyt *c* settled rapidly and was collected on a column. Cyt *c* was then purified according to the procedure of Sels *et al.* (23). The Cyt *c* migrated as a single band in dodecyl sulfate-polyacrylamide gel electrophoresis (14). An aliquot of purified reduced Cyt *c* had a 550/280 absorbance ratio of 1.23. The Cyt *c* (which was oxidized at 80%) was stored frozen in 10 mM Tricine-NaOH buffer (pH 7).

Binding Assays. The incubation medium used for binding assays contained 0.3 M sorbitol and 1 mM Tricine-NaOH buffer (pH 7.2) (standard medium). Binding assays with the labeled protein ^{59}Fe-Cyt *c* were carried out in a series of tubes containing the standard medium and increasing concentrations of the labeled protein. The incubation was started by addition of intact chloroplasts or membranes (envelopes or thylakoids) (total volume 4.5 ml). After an incubation period for 5 min at 0 C, the

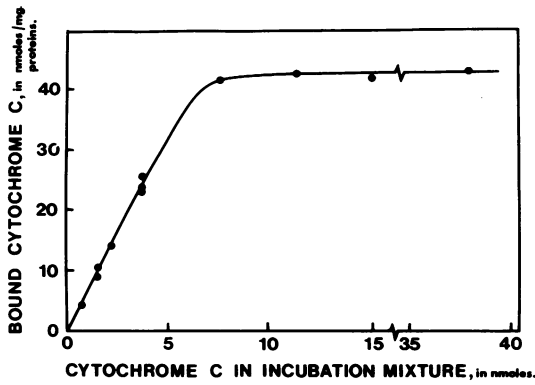

FIG. 1. Binding of Cyt *c* on the envelope of spinach chloroplast as a function of Cyt *c* in the incubation medium. Experimental procedure was described in the text.

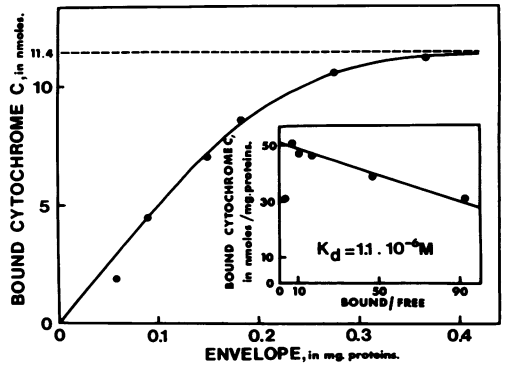

FIG. 2. Binding of Cyt *c* on the envelope of spinach chloroplast as a function of envelope proteins in the incubation medium. Experimental procedure was described in the text.

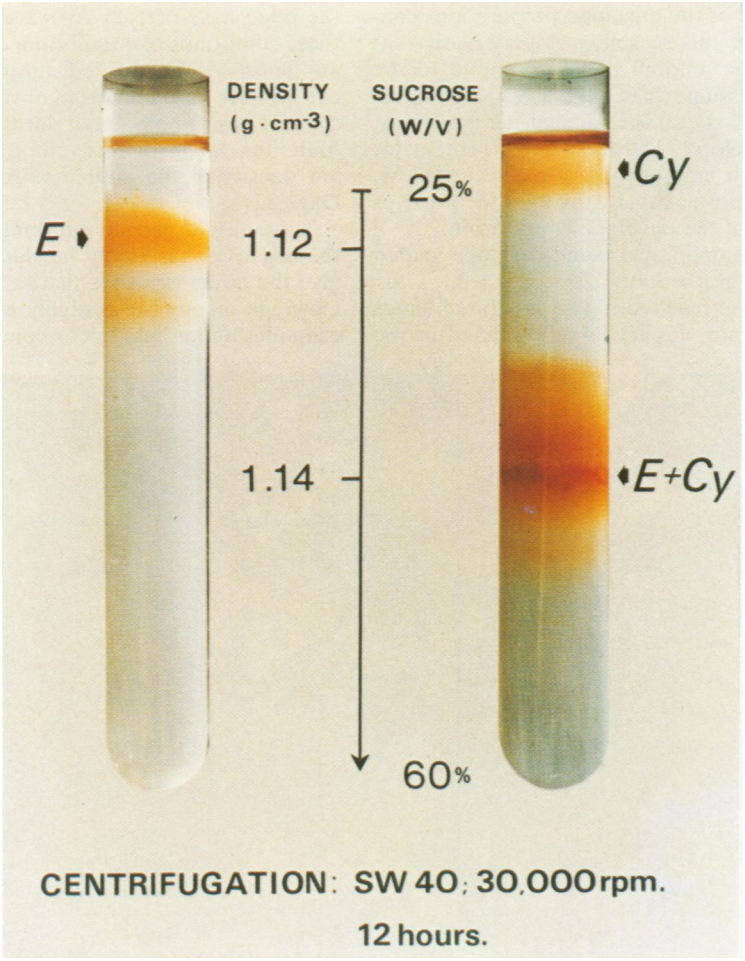

FIG. 3. Centrifugation of envelope fraction (left tube) and envelope loaded with Cyt *c* (right tube) on a discontinuous sucrose gradient.

suspensions were centrifuged. The incubation conditions were sufficient to allow equilibrium between bound and free ligands. The radioactivity in the pellets and the supernatants was determined by gamma counting (multichannel analyzer, Intertechnique SA 40 B, well-type NaI (T1) gamma ray detector).

Protein Determination. Protein content was determined by the method of Lowry *et al.* (17) with crystallized BSA as a standard.

Table I. Effect of Mg^{2+} on the Binding of Cytochrome C to the Envelope of Spinach Chloroplast

A suspension of chloroplast envelope (205 µg protein) was incubated in the standard medium (see text) containing a fixed amount of ^{59}Fe - Cyt C (15 nmol, 18,000 dpm mg^{-1} protein). After an incubation period for 5 min at 0 C, Mg^{2+} was added to the reaction mixture. The membranes were then collected by centrifugation as described in the text. Mg^{2+} added in the incubation medium before Cyt C gave identical results.

Mg^{2+} in the incubation medium mM	0	5	10	100
Bound Cyt C nmol	12.0	7.6	4.3	0.8

RESULTS AND DISCUSSION

Electron micrographs of the purified envelope fractions show single and double membrane vesicles of variable sizes with a cross-section width in the range of 60 to 80 10^{-8}cm (13, 25). No plastoglobuli are trapped in the network of the envelope membranes, no stroma lamellae are observed, and the Chl content is less than 0.1 µg/mg total membrane proteins. In good agreement with Sprey and Laetsch (25), we have observed in all cases that the envelope preparation obtained from unpurified chloroplasts is more or less contaminated by other chloroplast or cell constituents. The use of purified intact chloroplasts minimized the dangers of cross-contamination (6, 13, 19). Finally, the envelope vesicles are unstable. They fragment into smaller vesicles with time.

Yeast Fe-Cyt *c* is able to bind to the chloroplast envelopes with a low affinity (Kd = 1.1 µM) (Figs. 1 and 2). The total amount of low affinity binding sites is of the order of 50 nmol Cyt *c* mg^{-1} protein (0.6 mg Cyt *c* mg^{-1} protein). This value is much higher than that found for the anionic inner mitochondrial membrane (20). For example, Vanderkooi *et al.* (26) have identified 3 nmol Cyt *c*-binding sites/mg of protein in Cyt *c*-depleted pigeon heart mitochondria. The value for the envelope is also much higher than that found for thylakoids (10 nmol Cyt *c* mg^{-1} protein). The binding of Cyt *c* to the chloroplast envelope membranes is readily reversed by 50 to 100 mM Mg^{+2} (Table I). These results indicate that binding of Fe-Cyt *c* to the envelope is electrostatic and that the envelope membranes are strongly neg-

atively charged. However, it has to be stressed that the sucrose layers used during the course of the envelope preparations contained 4 mM $MgCl_2$ (13). For this reason, it is very likely that such assays underestimate the amount of Cyt *c* bound to the isolated membranes. If we assume that only one charge is required to bind 1 molecule of Cyt *c*, it is clear that the amount of sulfolipid present in the envelope (110 nmol mg^{-1} protein [6, 18]) is sufficient to bind such amount of Cyt *c*. Nevertheless, whether the envelope-Cyt *c* complex really involves the proteins and/or the lipid compounds of the envelope is uncertain.

Centrifugation of pure envelope fraction and envelope loaded with Cyt *c* on a discontinuous gradient of 0.73, 0.88, 1.02, 1.11, 1.20, 1.29, and 1.46 M sucrose resulted in two separated bands (Beckman SW 40, 30,000 rpm, 12 hr): a yellow band at the interphase of 0.73 to 0.88 M sucrose layers, and a red band at the interphase of 1.11 to 1.2 M sucrose layers (Fig. 3). Under these conditions of equilibrium density centrifugation, the buoyant density of the purified chloroplast envelope fraction (1.12 g/cm^3) differs markedly from envelope loaded with Cyt *c* (1.14 g/cm^3). It has to be pointed out that the buoyant density we have found for the purified envelope fraction is identical to the buoyant density of the double vesicles described by Poincelot and Day (22).

These results give no information about the precise location of the negative charges on the chloroplast envelope. However, we give the direct evidence that at least the outer face of the outer envelope membrane is highly negatively charged. Thus, if one examines under light microscopy the washed intact chloroplasts,

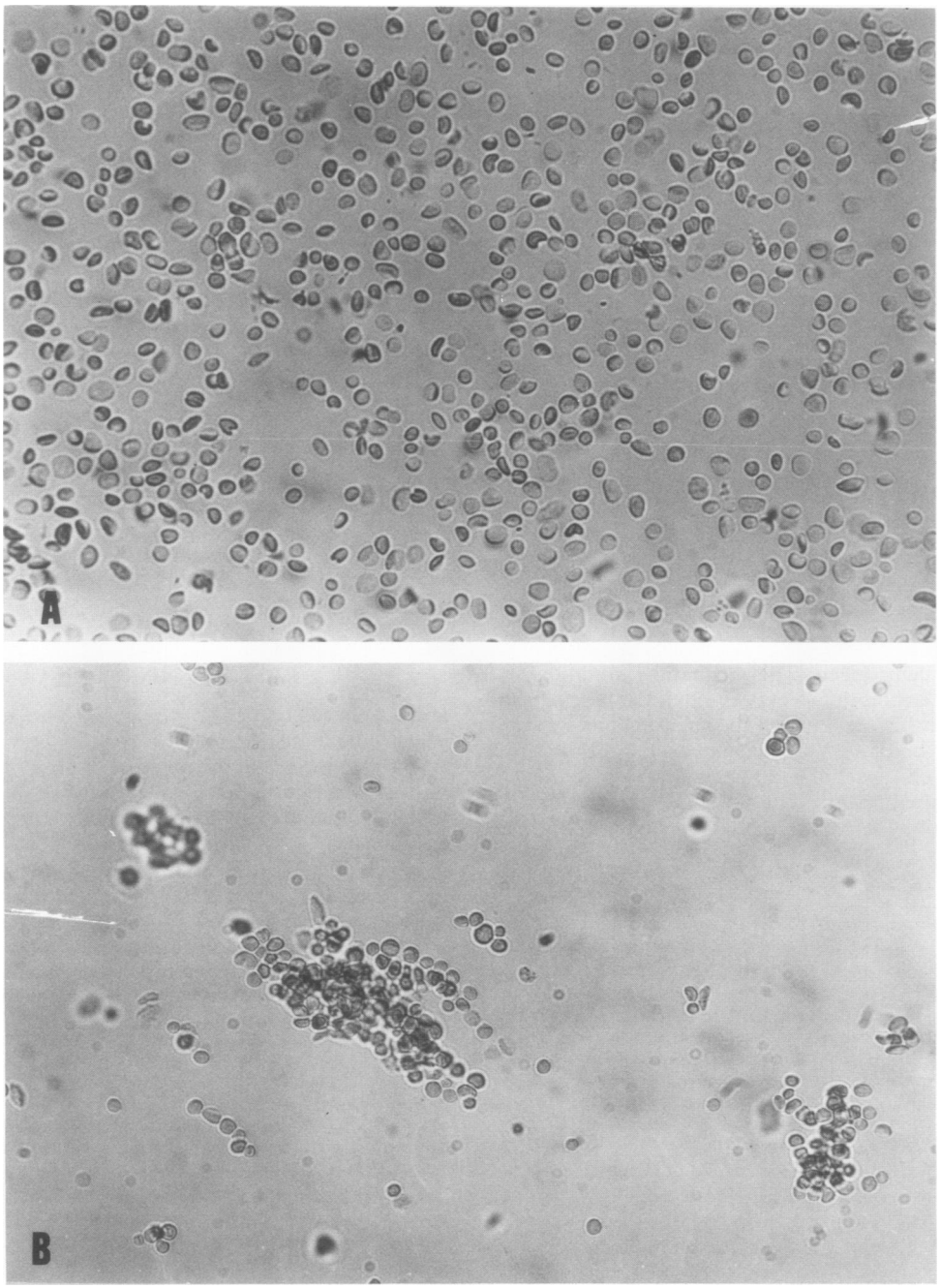

FIG. 4. Appearance of intact washed chloroplasts under light microscopy; A: without yeast Cyt *c*; B: with yeast Cyt *c* (50 μM). Preparation of intact washed chloroplasts and suspension medium were described under "Materials and Methods." In this particular experiment, the chloroplasts were 89% intact.

they look uniformly distributed through the preparation (Fig. 4A; ref. 16). Addition of yeast Cyt *c* (50 μM) to the preparation of intact washed chloroplasts induces a strong and rapid agglutination (Fig. 4B) of the chloroplasts. In this case, the amount of yeast Fe-Cyt *c* bound to the washed intact chloroplasts, corrected for the contribution of the broken thylakoids (16) is considerable (53 nmol mg^{-1} Chl). Addition of (2 mM) Mg Cl_2 to the medium prevents the agglutination of chloroplasts. The Cyt *c* molecule is a 34 × 34 × 30 Å prolate spheroid (5), and most of the hydrophylic lysines which carry positive charges are distributed on the major axis of the molecule (5). Inevitably, in these conditions, once bound to a chloroplast, the Cyt *c* still carries free positive charges which, in turn, are able to interact with other chloroplasts. This explains the strong and fast agglutination observed. Preliminary studies conducted with polycationic ferritin (N,N-dimethyl-1,3-propane-diamine conjugate of ferritin [4, 9]) led to the same conclusion.

Chloroplast envelopes consist of two osmiophilic membranes. It is evident that fixed anionic surface charges may play an important role: first in the recognition or interaction between the two envelope membranes; second in the transport of anion (7, 11, 27) and protein (12) such as small subunit of D-ribulose-1,5-bisphosphate carboxylase (3, 21), and third, in the interactions between the chloroplasts and the other cell organelles (8).

Acknowledgments – We would like to thank A. A. Benson and P. Vignais for their kind interest and helpful comments.

LITERATURE CITED

1. Akerlund HE, B Andersson, H Westrin, PA Albertsson 1975 Isoelectric point of chloroplast and subchloroplast fractions determined by cross partition. Abstr No 1028. 10th Meeting Fed Eur Biochem Soc Paris
2. Billecocq A 1975 Structure des membranes biologiques. Localisation du sulfoquinovosyl diglyceride dans les diverses membranes des chloroplastes au moyen des anticorps spécifiques. Ann Immunol (Inst Pasteur) 126C: 337-352
3. Cobb AA, AR Wellburn 1976 Polypeptide binding to plastid envelopes during chloroplast development. Planta 129: 127-132
4. Dannon D, L Goldstein, Y Marikovsky, E Skutelsky 1972. Use of cationized ferritin as a label of negative charges on cell surfaces. J Ultrastruct Res 38: 500-510
5. Dickerson RE 1972 The structure and history of an ancient protein. Sci Am 226: 58-72
6. Douce R, RB Holtz, AA Benson 1973 Isolation and properties of the envelope of spinach chloroplasts. J Biol Chem 248: 7215-7222
7. Flügge UI, HW Heldt 1976 Identification of a protein involved in phosphate transport of chloroplasts. FEBS Lett 68: 259-262
8. Gunning BES, MW Steer 1975 *In* E Arnold, ed, Ultrastructure and the Biology of Plant Cells. Crane Russak & Co, New York
9. Hackenbrok CR, KJ Miller 1975 The distribution of anionic sites on the surfaces of mitochondrial membranes. Visual probing with cationic ferritin. J Cell Biol 65: 615-630
10. Heber U 1973 Stoichiometry of reduction and phosphorylation during illumination of intact chloroplasts. Biochim Biophys Acta 305:140-152
11. Heber U 1974 Metabolite exchange between chloroplasts and cytoplasm. Annu Rev Plant Physiol 25: 393-421
12. Joy KW, RJ Ellis 1975 Protein synthesis in chloroplasts. IV. Polypeptides of the chloroplast envelope. Biochim Biophys Acta 378: 143-151
13. Joyard J, R Douce 1976 Préparation et activités enzymatiques de l'enveloppe des chloroplastes d'épinard. Physiol Vég 14: 31-48
14. Laemmli UK 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685
15. Leech RM 1964 The isolation of structurally intact chloroplasts. Biochim Biophys Acta 79: 637-639
16. Lilley RMcC, MP Fitzgerald, KG Rienits, DA Walker 1975 Criteria of intactness and the photosynthetic activity of spinach chloroplast preparations. New Phytol 75: 1-10
17. Lowry OH, NJ Rosebrough, AL Farr, RJ Randall 1951 Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265-275
18. Mackender RO, RM Leech 1974 The galactolipid, phospholipid and fatty acid composition of the chloroplast envelope membranes of *Vicia faba* L. Plant Physiol 53: 496-502
19. Morgenthaler JJ, L Mendiola-Morgenthaler 1976 Synthesis of soluble, thylakoid and envelope membrane proteins by spinach chloroplasts purified from gradients. Arch Biochem Biophys 172: 51-58
20. Nicholls P 1974 Cytochrome *c* binding to enzymes and membranes. Biochim Biophys Acta 346: 261-310
21. Pineau B, R Douce 1974 Analyse electrophorétique des protéines de l'enveloppe des chloroplastes d'épinard. FEBS Lett 47: 255-259
22. Poincelot RP, PR Day 1974 An improved method for the isolation of spinach chloroplast envelope membranes. Plant Physiol 54: 780-783
23. Sels AA, H Fukuhara, G Péré, PP Slonimsky 1965 Cinétique de la biosynthèse induite de l'Iso-1-cytochrome *c* et de l'Iso-2-cytochrome *c* au cours de l'adaptation à l'oxygène. Biochim Biophys Acta 95: 486-502
24. Sherman F, H Taber, W Campbell 1965 Genetic determination of Iso-cytochrome *c* in yeast. J Mol Biol 13: 21-39
25. Srey B, WM Laetsch 1975 Chloroplast envelopes of *Spinacia oleracea* L. I. Polypeptides of chloroplasts envelopes and lamellae. Z Pflanzenphysiol 75: 38-52
26. Vanderkooi J, M Erecinska, B Chance 1973 Cytochrome *c* interaction with membranes. II. Comparative study of the interaction of *c* cytochromes with the mitochondrial membrane. Arch Biochem Biophys 157: 531-540
27. Walker DA 1974 Chloroplasts and cells. The movement of certain key substances, etc. across the chloroplast envelope. *In* DH Northcote, ed, MTP International Review of Science. Biochemistry Series I Vol 11 Plant Biochemistry. Butterworths, London and University Park Press, Baltimore pp 1-49
28. Westrin H, P Albertsson, G Johansson 1976 Hydrophobic affinity partition of spinach chloroplasts in aqueous two-phase systems. Biochim Biophys Acta 436: 696-706