Composition and Spectral Properties of a Cytochrome-Enriched Fraction Obtained from Chloroplasts by Digitonin Treatment

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A cytochrome-enriched fraction obtained from chloroplasts after treatment with the detergent digitonin contained cytochromes *f*, *b*-559_{LP} and *b*-563 in the approximate proportions 1:1:2, close to those observed in unfractionated chloroplasts. The spectrum of cytochrome *b*-563 at temperature of liquid N₂ showed a single asymmetrical α -band with a maximum at 561 nm.

Wessels (1966) and Wessels & Voorn (1972) reported that, after treatment of chloroplasts with the detergent digitonin under suitable conditions and centrifugation of the resulting extract through a density gradient, a pink band was obtained in addition to various chlorophyll-containing fractions. This pink band was reported to contain cytochromes *f* and '*b*₆'. Nelson & Neumann (1972) described the preparation of a particle containing cytochromes *f* and *b*-563 starting with an initial digitonin treatment as described by Wessels (1966) and Wessels & Voorn (1972). Anderson & Boardman (1973) reported that a less-purified fraction obtained by such treatment contained cytochrome *b*-559_{LP} (LP indicates low potential), in addition to cytochromes *f* and *b*-563. Their evidence for the presence of the cytochrome included the observation of two bands attributable to *b*-type cytochromes in low-temperature difference spectra. This experimental finding was confirmed by Wood & Bendall (1976); however, Stuart & Wasserman (1973) reported that purified cytochrome *b*-563 had a split α -band at 77 K, so the interpretation could be questioned. Anderson & Boardman (1973) also provided some evidence that two spectrally distinct components could be separated kinetically during reduction with dithionite. A similar kinetic distinction of the low-potential cytochromes (*b*-559_{LP} and *b*-563) is possible in unfractionated chloroplasts (Bendall *et al.*, 1971), and at present this represents the most satisfactory way of measuring the two components. Some workers have used ascorbate-reduced minus quinol (hydroquinone)-reduced difference spectra to measure cytochrome *b*-559_{LP}. This is not satisfactory because ascorbate reacts slowly and incompletely with this cytochrome (Bendall *et al.*, 1971). The spectra reported by Anderson & Boardman (1973) suggested that cytochrome *b*-563 did not possess a split α -band at 77 K, in contrast with the report by Stuart & Wasserman (1973) for the purified component.

A fraction containing *b*- and *c*-type cytochromes has obvious analogies with complex III of the mitochondrial electron-transport chain, the ubiquinol-cytochrome *c* reductase. Wood & Bendall (1976) showed that the material from chloroplasts was capable of plastoquinol-1-plastocyanin oxidoreductase activity. The experiments described in the present paper were carried out to remove the ambiguity that exists with regard to the composition of the cytochrome-enriched fraction and the spectral properties of cytochrome *b*-563 at the temperature of liquid N₂.

Materials and Methods

Chloroplasts were isolated from spinach (*Spinacea oleracea*) and stored in liquid N₂ as described previously (Olsen & Cox, 1979). The cytochrome-enriched fraction was prepared from the thawed chloroplasts essentially by following the early stages of the procedure of Nelson & Neumann (1972). The chloroplast suspension was diluted with an equal volume of digitonin suspension to give final concentrations of 1.0 mg of chlorophyll/ml and 1.25% (w/v) digitonin in a medium containing 0.4 M-sucrose, 0.1 M-NaCl, 1 mM-MgCl₂, 10 mM-Tricine {*N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine}/NaOH buffer, pH 8.0, and 2.5% (v/v) dimethyl sulphoxide. The suspension was homogenized in a Potter-Elvehjem homogenizer and incubated at 4°C in the dark with gentle stirring for 16–20 h. Protamine sulphate was then added to a final concentration of 0.2 mg/ml. After a further 60 min incubation the suspension was centrifuged at 35000g for 30 min, and the yellow-green supernatant was dialysed against 0.1 M-potassium phosphate buffer, pH 6.5, and then stored frozen in liquid N₂.

All spectral measurements were taken in 0.1 M-potassium phosphate buffer, pH 6.5. Spectra at room temperature were taken by using a Cary 118C

spectrophotometer equipped with a scattered-transmission accessory. Spectra at the temperature of liquid N₂ were taken by using the low-temperature attachment of a Perkin-Elmer 356 spectrophotometer.

Results and Discussion

Quinol-reduced minus ferricyanide-oxidized difference spectra of the cytochrome-enriched fraction revealed the presence of cytochrome *f* but no evidence for the presence of a quinol-reducible cytochrome *b*-559, i.e. *b*-559_{HP} (HP indicates high potential). Fig. 1 shows difference spectra in the Soret and α -band regions at different times after the addition of 2 mM-dithionite to a sample previously reduced with quinol. A rapidly reduced component with maxima at about 430 nm and 559 nm can be distinguished from a slowly reduced component with maxima at 431 nm

and 563 nm. This demonstrates the presence of both cytochromes *b*-559_{LP} and *b*-563 in the preparation. A kinetic analysis of the changes at 561 nm (where the two cytochromes might be expected to have the same absorption coefficient) showed that the slow change

Table 1. Cytochrome contents of the cytochrome-enriched fraction

The cytochrome concentrations were measured as described in the text.

Preparation	Relative cytochrome content (mol/mol)		
	<i>f</i>	<i>b</i> -559 _{LP}	<i>b</i> -563
1	1.0	0.9	2.0
2	1.0	1.0	1.8

Fig. 1. Difference spectra of cytochrome-enriched fraction at room temperature

Trace (a), baseline. Trace (b), immediately after the addition of dithionite (2 mM) to the sample cuvette; both cuvettes contained 1.7 mM-quinol and a trace of catalase. The peak was scanned at 30 s (α -band region) or 40 s (Soret-band region) after the addition of dithionite. Trace (c), the final difference spectrum taken after 20 min. Trace (d), calculated difference spectrum obtained by subtracting spectrum (b) from spectrum (c). The spectrum in the Soret region was taken with a sample twice as dilute as that used for the α -band region. Other details were as described in the Materials and Methods section.

Fig. 2. Difference spectra of the cytochrome-enriched fraction at 77K

To obtain the spectrum shown by the continuous line, dithionite to a final concentration of 2mM was added to the sample and allowed to react for 15 min at room temperature. The same concentration of dithionite was then added to the reference compartment, and the low-temperature cuvette was immediately placed into liquid N₂. The broken line represents the difference spectrum between a sample oxidized with ferricyanide (1.7mM) and a reference reduced with ascorbate (1.7mM) taken under the same instrument settings. Other details were as described in the Materials and Methods section.

represented 58% of the total. Assuming similar absorption coefficients for the three cytochromes at their α -band maxima, proportions of about 1:1:2 for cytochromes *f*, *b-559_{LP}* and *b-563* in the cytochrome-enriched material were found (Table 1). These are close to the proportions found in un-

fractionated chloroplasts (Cox & Bendall, 1972; Heber *et al.*, 1976).

Fig. 2 shows a difference spectrum at the temperature of liquid N₂ (77K) in which the sample had been incubated with 2mM-dithionite for 15min before being frozen and the reference had been frozen immediately after the addition of the same concentration of dithionite. The spectrum is thus solely that of the slowly reduced cytochrome *b-563*. Although the α -band is asymmetrical, there is no evidence for a split peak. Measurements of cytochrome *f* under the same instrument settings confirmed that the resolution of the apparatus was adequate to resolve the split α -band of this cytochrome. This result agrees with the report by Anderson & Boardman (1973). It is possible that the split α -band observed by Stuart & Wasserman (1973) is an artifact resulting from a proportion of their material being altered as a result of the extremely rigorous extraction conditions used; these included the use of Triton X-100, and it has been reported that this detergent can alter the spectral properties of purified cytochrome *b-563* (Nelson & Neumann, 1972).

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