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The purification of cytochrome *f* and plastocyanin using affinity chromatography

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Both plastocyanin and cytochrome *f* were purified using a combination of affinity chromatography together with established methods. Plastocyanin was partially purified using the method of Davis and San Pietro (Anal. Biochem. 95 (1979) 254–259), after which it was further purified using a column of cytochrome *c* covalently attached to Sepharose 4B. The affinity column was prepared using the method of Godinot and Gautheron (Methods Enzymol. 54 (1979) 112–114). The final purity index ratio (A_{278}/A_{597}) was less than 1.2, which is equal to that obtained using the more expensive FPLC procedure (Anderson, G.P., Sanderson, D.G., Lee, C.H., Durell, S., Anderson, L.B. and Gross, E.L. (1987) Biochim. Biophys. Acta 894, issue 3). Cytochrome *f* was partially purified using a modification of the method of Matazaki et al. (Plant Cell. Physiol. 16 (1975) 237–246) and bound to an affinity column of plastocyanin covalently attached to Sepharose 4B. Cytochrome *f* purified using this procedure had a purity index ratio ($A_{554.5}/A_{277}$) of 1.2. Both proteins are tyrosine proteins containing no tryptophan residues. After the affinity chromatography step, the fluorescence emission spectrum of either plastocyanin or cytochrome *f* was typical of a tyrosine protein free from tryptophan contamination.

Introduction

Cytochrome *f* and plastocyanin are electron transport partners functioning between Photosystem II and Photosystem I in the Z-scheme of photosynthesis [1,2]. Cytochrome *f* is a part of cytochrome b_6f, which also contains cytochrome b_6, a Rieske iron sulfur protein, a bound quinone and a 17 kDa protein of unknown function. The movement of protons into the thylakoid lumen is coupled to the passage of electrons through cytochrome b_6f in both the linear and cyclic electron transport of higher plants. Plastocyanin is a 10.5 kDa copper-containing protein [3] which functions as a redox intermediate between cytochrome b_6f and Photosystem I. Electrons are transferred from cytochrome *f* to plastocyanin on the inner surface of the thylakoid [4]. The plastocyanin is oxidized by the reaction center of Photosystem I and cytochrome *f* is rereduced by the Rieske iron-sulfur protein [5]. Recent studies using limited proteinase digestion of inside-out and right-side-out thylakoid vesicles have revealed that the four major polypeptides of cytochrome b_6f are transmembrane, with the carboxyl-ends exposed to the stroma [6,7].

The functional groups of cytochrome *f* involved in electron transfer and interaction with its reaction partners are not defined. The altered kinetics of reactions using either chemically modified cytochrome *f* [8,9] or plastocyanin [10] have revealed the importance of local positive charges

Abbreviations: CM-Sephadex, carboxymethyl-Sephadex; FPLC, fast protein liquid chromatography; PC-Sepharose, plastocyanin covalently attached to Sepharose 4B.

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on cytochrome *f* for interaction with plastocyanin. Although both proteins have a net negative charge [10], the modification results show that cytochrome *f* has 'local positive charges' at the binding site for plastocyanin.

Plastocyanin has been shown to have two distinct sites for interaction with redox reagents [11,12]. Negatively charged donors bind at the 'north site' near the His-87 ligand to the copper, while positively charged molecules bind at the more remote or 'east site' near tyrosine 83, which is surrounded by negatively charged amino acids. Recent reports [13,14] support the hypothesis that the 'east site' is the binding site for cytochrome *f*.

In order to study the spectroscopic properties of proteins such as cytochrome *f* and plastocyanin, it is necessary for the proteins to be very pure. This is particularly true for fluorescence studies, since both proteins are tyrosine proteins containing no tryptophan residues. Since tryptophan has a much higher fluorescence yield than tyrosine [15], small amounts of tryptophan-containing contaminants will alter the shape of the fluorescence spectrum.

In this paper, we report the use of affinity chromatography for the isolation of both plastocyanin and cytochrome *f*. A plastocyanin-affinity column using plastocyanin covalently attached to Sepharose was used to isolate cytochrome *f*. Plastocyanin is known to form a complex with cytochrome *f* as discussed above. An affinity column with cytochrome *c* covalently attached to Sepharose was used to isolate plastocyanin. The rationale for this is that NMR studies have shown that plastocyanin forms a redox active complex with cytochrome *c* [16].

Materials and Methods

Preparation of affinity chromatography stationary phase. Cytochrome *c* or plastocyanin was covalently attached to cyanogen bromide activated Sepharose 4B as described by Godinot and Gautheron [17]. The cytochrome *c* was obtained from Sigma Chemical Co. and used without further purification. The PC-Sepharose was prepared using 10 mg plastocyanin with 3 g (dry) of cyanogen bromide activated Sepharose. The plastocyanin attached to the affinity column is redox-active in that it can be reduced with ascorbate and oxidized with ferricyanide. It retained both its redox activity and binding affinity for months.

Purification of plastocyanin. Turnip plastocyanin was purified according to Davis and San Pietro [18]. After a single Sephadex G-75 column, the plastocyanin had a purity index ratio (A_{278}/A_{597}) for the oxidized form of 1.4. The plastocyanin was further purified using the cytochrome *c*-Sepharose affinity column. This column was equilibrated with 10 mM phosphate buffer (pH 7.0). The plastocyanin was applied to the column in the oxidized state and then eluted with phosphate buffer (pH 7.0) in steps from 10 to 100 mM.

Purification of cytochrome f. Turnip cytochrome *f* was purified using the method of Matsuzaki et al. [19]. This procedure was followed through the acetone precipitation step with the exception that 3 kg of turnip leaves were homogenized with 500 ml of 40 mM phosphate (pH 7.0) + 2 mM EDTA and 1 liter of ethylmethyl ketone in the initial step. The acetone pellet was resuspended in 25 mM phosphate (pH 7.0) and dialyzed against the same buffer overnight.

Treatment at pH 5 and hydroxyapatite chromatography. The material was then centrifuged at $37\,000 \times g$ for 20 min, and the pH of the supernatant was adjusted to 5.0 using 1 M HCl. After stirring for 20 min and a second centrifugation, the resulting supernatant was returned to pH 7.0 using 1 M NaOH and applied to a hydroxyapatite column which had been previously equilibrated with 25 mM phosphate (pH 7.0), 100 mM NaCl. The column was washed with the equilibration buffer until the eluate was colorless and then the bound material was eluted with 300 mM phosphate (pH 7.0).

CM-Sephadex chromatography. The brownish red material obtained from the hydroxyapatite column was dialyzed against 10 mM succinate (pH 5.0) overnight, centrifuged and applied to CM-Sephadex which had been equilibrated with 10 mM succinate (pH 5.0). After washing with 50 ml of 10 mM succinate, the bound material was eluted with 50 ml of 100 mM phosphate (pH 7.0). The orange fractions were concentrated in an Amicon cell using a YM30 membrane, passed through a P-10 column equilibrated with 10 mM

phosphate (pH 7.0) and then applied directly to a DEAE-cellulose column equilibrated with 10 mM phosphate (pH 7.0). This column was washed with 100 ml of 10 mM phosphate then was eluted with 20 mM phosphate (pH 7.0).

PC-Sepharose chromatography. The colored fractions were concentrated as above and passed through a P-10 column equilibrated with 5 mM phosphate (pH 6.0) and then applied to the PC-Sepharose column. After washing with 50 ml of 5 mM phosphate, the cytochrome *f* was eluted with 50 mM phosphate (pH 6.0).

Absorption and fluorescence measurements. Absorption spectra of plastocyanin and cytochrome *f* were measured using an Aminco Model DW 2a spectrophotometer in the dual beam mode. Fluorescence emission spectra were measured using an SLM Aminco SPF-500C spectrofluorometer. The excitation wavelength was 280 nm and the slit widths were 5 nm.

Results

Purification of plastocyanin

When the cytochrome *c*-Sepharose column was eluted using phosphate buffer (Fig. 1a), there was clear separation of plastocyanin from the contaminants. In contrast, little separation was observed when Tris buffer (pH 8.2) was used (Fig. 1b). Phosphate ions are known to bind specifically to cytochrome *c* [20] and this may be involved in its function as an affinity column.

TABLE I

PURIFICATION OF CYTOCHROME *f*

Purification was carried out as described in the Materials and Methods section.

Procedure	Cytochrome *f* (nmol)	Purity ($A_{554.5}/A_{277}$) ($\times 10^2$)	Percent yield
Initial extraction	–	0.084	–
Ammonium sulfate	–	0.079	–
Acetone precipitate	310	4	100
Treatment at pH 5	250	4	81
Hydroxyapatite	180	10	58
CM-Sephadex	120	30	40
DEAE-cellulose	70	80	23
PC-Sepharose	20	120	6

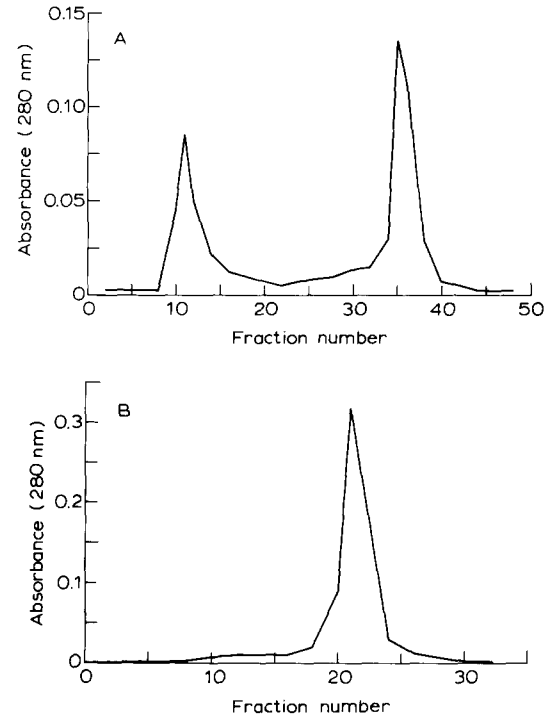

Fig. 1. The elution profile for the purification of plastocyanin using cytochrome *c*-Sepharose. (A) Elution with phosphate buffer (pH 7). Plastocyanin is eluted in fractions 30 to 40 (see text). (B) Elution with Tris (pH 8.2). Partially purified plastocyanin was applied to column equilibrated with 10 mM Tris. A Tris step gradient (10 mM steps) was used to elute the sample. Plastocyanin was not separated from contaminants.

Oxidized plastocyanin, applied to the column, was eluted in the reduced state demonstrating electrochemically active binding. The final purity index ratio (A_{278}/A_{597}) of 1.12 is equivalent to that obtained using the more expensive FPLC procedure [21].

Fluorescence emission spectra were used as the final test of purity. The fluorescence emission spectrum of the plastocyanin prior to and after purification on the affinity column is shown in Fig. 2a. A typical tyrosine fluorescence spectrum has an emission maximum at 305 nm. In contrast, tryptophan, in protein, has a fluorescence maximum between 320 and 340 nm and has a much higher quantum yield [15]. It is observed that the tryptophan contaminants have been removed. The fluorescence emission spectrum is a more sensitive test of purity than is the absorption ratio.

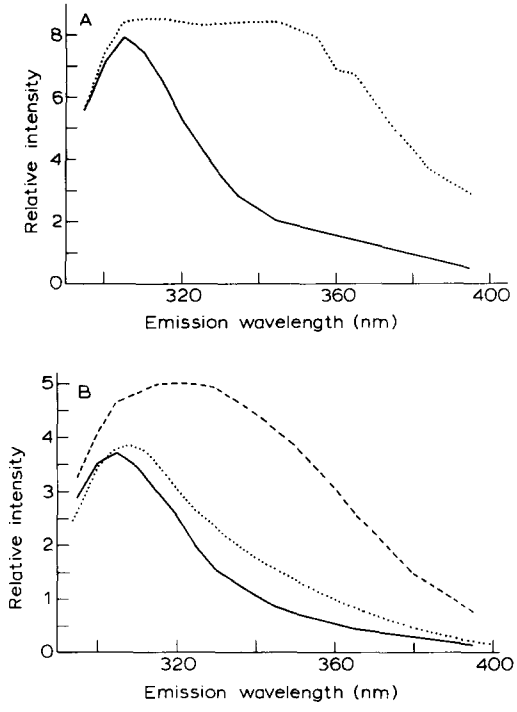

Fig. 2. The near-ultraviolet fluorescence emission spectra of plastocyanin and cytochrome *f*. (A) Plastocyanin (10 μM). Before (- - - - - -) and after (———) cytochrome *c*-Sepharose chromatography. (B) The $A_{554.5}/A_{277} = 0.6$ (- - - - - -) and 1.2 (———) samples are from the same preparation and represent cytochrome *f* before and after PC-Sepharose purification, respectively. The dotted line (· · · · · ·) sample had a purity index ratio of 1.0. The spectra are corrected to 10 μM cytochrome *f*, dynode voltage = 660 V and signal gain = 100.

Purification of cytochrome f

A typical purification profile of turnip cytochrome *f* is presented in Table I. Cytochrome *f* was not detected, using reduced minus oxidized absorbance at 554.5 nm, after the first two steps of purification. Thus, the total yield could be calculated only after acetone precipitation, and percentage yield was calculated relative to this step. Since the mass of the acetone pellet was directly proportional to the amount of material lost by isoelectric precipitation at pH 5, the latter purification step is required to insure reproducibility in the column chromatography steps. The values in Table I for PC-Sepharose purified cytochrome *f* were obtained from a single 3 ml fraction. A total of 50 nmol of cytochrome *f* were eluted representing a total yield of 16%. The purity ($A_{554.5}/A_{277}$) of the other fractions ranged from 0.8 to 1.1.

Fig. 2b shows the fluorescence emission spectrum of cytochrome *f* before and after PC-Sepharose purification (see legend). Before affinity chromatography, there is a significant amount of tryptophan contamination in the sample. PC-Sepharose purified cytochrome *f* has a typical tyrosine-protein fluorescence spectrum, free from tryptophan contamination.

The purity index ratio ($A_{554.5}/A_{277}$) obtained after affinity chromatography is 1.2 compared to 0.65–0.83 reported by Matsuzaki et al. [19]. More important, however, is the change in the fluorescence emission spectrum, since samples of cytochrome *f* isolated using the Matsuzaki procedure showed significant tryptophan fluorescence.

Visible and near-ultraviolet absorption spectra of cytochrome *f* purified by affinity chromatography are shown in Fig. 3. They are similar to those previously reported [19,22].

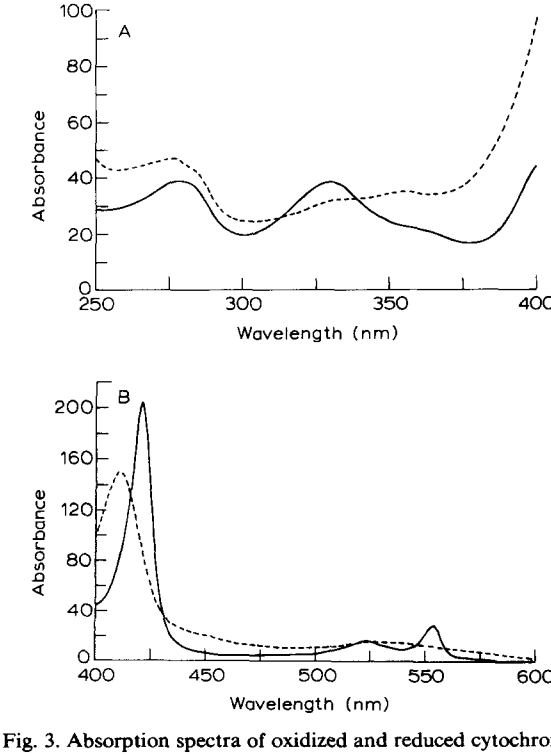

Fig. 3. Absorption spectra of oxidized and reduced cytochrome *f*. Corrected to 1 mM cytochrome *f*. - - - - - -, oxidized; ———, reduced. (A) Near-ultraviolet and (B) visible.

Discussion

Since cytochrome *c* has a net positive charge (p*I* 10.6) [23], whereas plastocyanin has a net negative charge [10], the possibility arises that separation occurs due to ion exchange rather than affinity chromatography. Two pieces of evidence suggest that the cytochrome *c*-Sepharose column is acting as a true affinity column.

First, cytochrome *c* is known to form a redox active complex with plastocyanin. King et al. [16] have characterized the plastocyanin-cytochrome *c* complex using NMR spectroscopy. Their findings indicate the formation of strong 1 : 1 redox active complexes with the negative 'east site' of plastocyanin interacting with the positive heme edge region of cytochrome *c*. The redox active region of cytochrome *c* may be limited to 0.6% of the total surface area of the molecule [23].

Second, the oxidized plastocyanin was reduced on the column, indicating that the plastocyanin binds to the column in the correct location and orientation to promote electron transfer. The observation that plastocyanin binds to the column in either Tris or phosphate buffer while the contaminants bind in Tris only also demonstrates the high affinity of plastocyanin for cytochrome *c*.

Although plastocyanin may interact with cytochrome *c* and cytochrome *f* in a similar manner, there is no doubt that the PC-Sepharose column acts as an affinity column, since both cytochrome *f* (p*I* 5.5) and plastocyanin (p*I* 4.5) are negatively charged at pH 6. Thus, binding occurs via a positively-charged recognition site. For this reason, the PC-affinity column may prove useful for the separation of chemically modified forms of cytochrome *f* modified at the binding site from those modified at other locations.

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