Trimeric structure and other properties of the chloroplast reductase binding protein

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A complex between the ferredoxin-NADP$^+$ reductase and a 17.5 kDa polypeptide was isolated from lettuce chloroplasts by the same procedure used previously in spinach [(1984) J. Biol. Chem. 259, 8048–8051]. The stoichiometry of the complex from both sources was determined by quantification of stained protein bands after gel electrophoresis and, in the case of the spinach complex, by rocket inmunolectrophoresis. The results suggest that the binding protein is a trimer of the 17.5 kDa polypeptide. The amino acid composition of the spinach reductase binding protein and partial sequencing of its tryptic peptides clearly show that it is not identical to the 17 kDa polypeptide of the cytochrome b_6-f complex.

Reductase binding protein *Cytochrome* b_6-f *complex* *Ferredoxin-NADP$^+$ reductase* *Thylakoid intrinsic polypeptide*

1. INTRODUCTION

Chloroplast ferredoxin-NADP$^+$ oxidoreductase (EC 1.18.1.2) is the final enzyme of the photosynthetic electron transport chain. It was first described as an NADPH-dependent diaphorase [1] and thoroughly studied in its soluble form. The enzyme is bound to the stroma surface of the thylakoid membrane probably involving an electrostatic mechanism [2].

The membrane-bound reductase can be distinguished from the purified flavoprotein by the following allotopic properties: (i) dansyl chloride did not inhibit the diaphorase activity of thylakoids [3], whereas it inactivated the soluble form [4]; (ii) the pH profile of the diaphorase activity of soluble reductase presents an alkaline shift of about 1 pH unit with respect to the membrane-bound enzyme [3,5]; and (iii) the thylakoid-bound flavoprotein, but not the soluble one, undergoes a light-induced conformational change that may be of physiological importance in its regulation [6]. Rebinding of the enzyme to depleted thylakoids showed the existence of a definite stoichiometry suggesting rather specific binding sites for the reductase [2].

Recently, the ferredoxin-NADP$^+$ reductase of spinach chloroplasts was purified from a Triton X-100 thylakoid extract closely associated with an intrinsic polypeptide of 17.5 kDa [7]. It was suggested that this integral polypeptide is the binding site for the reductase and that it may play an important role in the physiological regulation of the flavoprotein.

Here we describe the isolation of a similar 17.5 kDa binding protein-reductase complex from lettuce leaves and determine the stoichiometry of this complex and of the complex obtained from spinach. Amino acid composition and partial sequencing of the spinach binding protein allow one to distinguish it from the 17 kDa component of cytochrome b_6-f complex.

2. MATERIALS AND METHODS

Isolation of the 17.5 kDa polypeptide-reductase

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complex and purification of the intrinsic component from lettuce or spinach thylakoids were performed as in [7].

SDS gel electrophoresis was carried out as described [8]. Densitometric traces were recorded with a Gilson Gel Scanning Holochrome at 600 nm. Quantitation of Coomassie blue and amido black staining was also performed by dye extraction essentially as in [9], using 30% pyridine. Both methods and dyes gave similar results. Protein concentration was determined according to [10].

Amino acid analysis after acid hydrolysis using *o*-phthalaldehyde as derivatizing agent was carried out by HPLC according to [11]. Sequence determination was performed as described in [12]. Tryptophan content was measured by the method of Sasaki et al. [13]. Cysteine content of the reductase binding protein was determined by measuring the incorporation of *N*-[^{3}H]ethylmaleimide. The polypeptide (1 mg/ml) was incubated at 25°C for 2 h in a medium containing 15 mM dithioerythritol, 1 mM EDTA, 2% SDS and 50 mM Tris-HCl, pH 7.8. Then, the samples were filtered twice through Sephadex G-50 equilibrated with 20 mM Mops, pH 7, 0.01% SDS and 1 mM EDTA by a centrifugal desalting method [14]. After incubation for another 3 h with 5 mM *N*-[^{3}H]ethylmaleimide the samples were filtered again, and assayed for protein concentration and radioactivity incorporated. Radioactivity was measured in a Beckman LD 8100 liquid scintillation counter using a mixture of 0.6% 2,5-diphenyloxazole, 30% Triton X-100 in toluene.

Tryptic digestion of the binding protein with 2% (w/v) TCK-treated trypsin was carried out for 24 h in 2% $(NH_4)CO_3H$. The HPLC assembly (Waters Associated, Millford, USA), used for peptide separation and amino acid analysis, consisted of 2 solvent delivery systems (M-6000 A and M-45), one N-660 solvent programmer, a U6K universal injector and a Gilson Holochrome spectrophotometer or Perkin-Elmer 650-40 fluorescence spectrophotometer.

Affi-gel blue and M_r standards were from Bio-Rad, *N*-[^{3}H]ethylmaleimide (9760 cpm/nmol) was purchased from New England Nuclear. Tris, *o*-phthalaldehyde, amido black, Coomassie blue, tryptophan and TCK-treated trypsin were from Sigma. All other reagents were of analytical grade.

3. RESULTS AND DISCUSSION

3.1. *Stoichiometry of the reductase-binding protein complex*

The ferredoxin-NADP$^+$ oxidoreductase associated to a 17.5 kDa polypeptide was isolated from lettuce chloroplasts by the same procedure described for spinach chloroplasts [7]. The purified complex subjected to SDS-polyacrylamide gel electrophoresis showed the same two components, namely the reductase and the 17.5 kDa band, when stained. Dye associated with protein bands from complexes obtained from both spinach and lettuce chloroplasts was quantitated, in the latter case by 2 different methods (table 1). The results show on average a stoichiometry of 3:1 (reductase binding protein: ferredoxin-NADP$^+$ reductase) with 4–6 different gels and preparations. It is noteworthy that similar values of stoichiometry were obtained for both spinach and lettuce preparations.

The stoichiometry was also determined by an independent method. The amounts of ferredoxin-NADP$^+$ reductase and binding protein present in spinach thylakoids per mg chlorophyll was determined by rocket immunoelectrophoresis [15]. Table 2 shows the results of 4 experiments. The average ratio of binding protein to reductase found was 3.05:1, in good agreement with the value reported above.

3.2. *Amino acid composition and partial sequencing of the reductase binding protein from spinach chloroplasts*

Analysis of the amino acid composition of the

Table 1

Stoichiometry of the binding protein-reductase complex

	Binding protein/reductase ratio		
	Densitometry	Dye extraction	
	Lettuce		Spinach
Amido black	3.08 (0.05)	2.84 (0.07)	2.90 (0.62)
Coomassie blue	3.13 (0.06)	3.30 (0.73)	3.20 (0.30)

The ratio was calculated from the values obtained by quantification of stained protein bands after SDS electrophoresis carried out as described in the text. Numbers in parentheses, SD (n = 4–6)

Table 2

Titration of the reductase and the binding protein in thylakoids by rocket immunoelectrophoresis

Expt no.	FNR/Chl (nmol/mg)	BP/Chl (nmol/mg)	BP/FNR
1	4.1	13.0	3.2
2	4.0	12.5	3.1
3	3.7	13.0	3.5
4	3.5	8.5	2.4

Reductase (FNR) or binding protein (BP) were quantified according to [18], using specific rabbit antibodies. Agarose gels contain 0.05% Triton X-100

17.5 kDa polypeptide from spinach chloroplasts (table 3) shows that about 50% of the residues are non-polar. Independent determination of cysteine and tryptophan residues was carried out because they could not be quantitated by the method used. About 0.85 mol *N*-[^{3}H]ethylmaleimide were incorporated per mol reductase binding polypeptide after dissociation and reduction of the protein, thus indicating the existence of a single cysteine residue per mol. One mol tryptophan per mol protein was found by the fluorometric method of Sasaki et al. [13].

Table 3

Amino acid composition of the reductase binding protein

Amino acid	Number of residues (nearest integer)
Asx	11
Glx	11
Ser	13
Gly	9
His	1
Thr	8
Ala	8
Arg	6
Tyr	6
Met	2
Val	12
Phe	6
Ile	10
Leu	16
Lys	6
Cys	1
Trp	1

This composition was determined as described in the text. Proline does not react with *o*-phthalaldehyde and was not determined

The minimum protein M_r calculated from the amino acid composition was 16838 in good agreement with that estimated by SDS-polyacrylamide gel electrophoresis (17500) [7].

A 17 kDa polypeptide was reported as a component of the cytochrome b_6-f complex isolated from spinach chloroplasts [16]. A slightly different method of isolation of the cytochrome complex yields a preparation with an additional 37 kDa polypeptide [17] which seems to be the reductase [18]. Considering these observations we have suggested [7] that the 17 kDa polypeptide from the cytochrome b_6-f complex might be the reductase binding protein isolated by us. Since the amino acid sequence of the 17 kDa polypeptide from the cytochrome b_6-f complex has been recently reported by Widger et al. [19], a comparison with the binding protein was possible and desirable. A confrontation of the amino acid composition of both polypeptides yielded significant quantitative differences. However, for a better comparison a further characterization of the reductase binding protein was convenient. Thus, it was subjected to tryptic digestion and the resulting peptides separated by HPLC (not shown). Five polypeptides were selected for further purification, amino acid composition, and partial sequencing (table 4). The peptides had between 5 and 10 residues each. The sequence of the pentapeptide was completed (peptide no.4, table 4). At the end of the third cycle an amino acid was not released, suggesting the presence of proline. According to the sequence reported [19] trypsinization of the 17 kDa polypeptide from the cytochrome b_6-f complex would yield 6 peptides of 64, 5, 24, 6, 27 and 7 residues and a free amino acid. After a careful search none of the 5 peptides isolated from the reductase binding protein corresponds to any of the 17 kDa polypeptide. Particularly, the pentapeptide (Thr-Val-Pro-Asn-Lys) was completely different from the pentapeptide from the binding protein (table 4). Thus, the reductase binding protein and the 17 kDa polypeptide of the cytochrome b_6-f complex are different proteins. Nevertheless, these results do not preclude a possible functional

Table 4

Tryptic peptides from the spinach binding protein

Peptide no.	Amino acid sequence or composition
1	Ala-Gly-(Asx, Glx_2, Ser, Thr, Val)-Arg
2	Glx-Val-(Asx, Ser_2, Gly_2, Ala, Val)-Arg
3	Ile-(Asx, Glx, Ser, Thr_2)-Arg
4	Asx-Gly-Pro-Leu-Arg
5	Leu-X-Phe-(Asx, Glx, Ser, Gly, Ile)-Arg

The peptides were subjected to manual Edman degradation or amino acid composition as described in section 2

interaction between the reductase binding protein complex and the cytochrome complex which would account for the proposed participation of the reductase in cyclic electron flow [20].

In summary, we postulate that the reductase is attached to the thylakoid membrane through a trimer of the 17.5 kDa intrinsic polypeptide. This binding protein has not chromophores or prosthetic groups detectable by absorption or fluorescence spectra. Its amino acid composition is in agreement with its intrinsic membrane location and it is different from the 17 kDa component of the cytochrome b_6-f complex.

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