Association of ferredoxin-NADP$^+$ oxidoreductase with the chloroplast cytochrome *b–f* complex

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The 37-kDa non-heme component in spinach cytochrome *b–f* complex prepared from EDTA-washed thylakoids [(1983) *J. Biol. Chem.* 258, 10348–10354] is shown to be ferredoxin-NADP+ oxidoreductase (EC 1.18.1.2) on the basis of immunoreactivity, amino acid analysis, and pattern of cleavage by cyanogen bromide. Strong binding of the reductase to the isolated cytochrome complex suggests this is an important site for its attachment to the thylakoid membrane in vivo.

Ferredoxin-NADP+ reductase *Cytochrome* b–f *complex* *Chloroplast* *Thylakoid*

1. INTRODUCTION

A protocol developed in this laboratory [1] for isolation of the spinach cytochrome *b–f* complex employs EDTA washes to deplete the thylakoids of CF_1. In contrast to washing with NaBr [2], EDTA leads to less extensive release of membrane-bound proteins; release of protein and pigments upon subsequent detergent extraction is also less extensive. Treatment with octyl glucoside and cholate [1] solubilizes a cytochrome *b–f* complex composed of a heme-containing polypeptide of 34 kDa (cytochrome *f*) and 4 other prominent polypeptides, including a non-heme component migrating at 37 kDa on SDS-polyacrylamide gel electrophoresis (SDS–PAGE).

Spinach ferredoxin-NADP$^+$ oxidoreductase has an apparent molecular mass in the range 34–38 kDa [3,4]. Here, we show that the 37-kDa polypeptide in spinach cytochrome *b–f* complex is, in fact, ferredoxin-NADP$^+$ oxidoreductase.

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Abbreviations: CF_1, the coupling factor 1 from spinach chloroplasts; PQ, plastoquinone; cyt, cytochrome; Tricine, *N*-tris(hydroxymethyl)methylglycine; FNR, ferredoxin-NADP$^+$ oxidoreductase

2. MATERIALS AND METHODS

Cytochrome *c–f* complex was prepared from spinach (*Spinacia oleracea* L. var. Hybrid 424) leaves as in [1]; this procedure was also applied to pea leaves (*Pisum sativum* L. var. Alaska) without modification. Unless stated otherwise, complex eluting from Biogel P300 was used without further purification. Ferredoxin was isolated by an established procedure [5]. Ferredoxin-NADP$^+$ oxidoreductase was purified from spinach by extraction at low ionic strength (in preparation).

^{125}I protein A was purchased from New England Nuclear; NADPH and equine heart cytochrome *c* were obtained from Sigma. Monospecific antibody to ferredoxin-NADP$^+$ oxidoreductase was a gift from N.-H. Chua, Rockefeller University.

Cytochrome *b–f* complex was assayed for PQ-1 : plastocyanin oxidoreductase activity at pH 6.5, ionic stregnth 0.1 M [1]. Ferredoxin-NADP$^+$ oxidoreductase activity was determined from the ferredoxin-dependent rate of cytochrome *c* reduction by NADPH. The standard assay mixture contained 30 μM cytochrome *c*, 3 nmol spinach fer-

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redoxin and 30 μM NADPH in 1.5 ml of 0.1 M Tris–Cl (pH 7.5). Cytochrome reduction was determined from the initial rate of increase in absorbance at 550 nm, using a reduced-minus-oxidized extinction coefficient of 18000 $M^{-1} \cdot cm^{-1}$.

SDS–PAGE was performed as in [6,7]. Electrophoretic transfer to nitrocellulose [8] was carried out for 75 min at 60 V, then 75 min more at 100 V, in a Hoefer Transphor TE-50. Immunological detection of ferredoxin-NADP$^+$ oxidoreductase on the nitrocellulose sheet was performed as in [9], using antiserum to the enzyme and ^{125}I protein A.

3. RESULTS

Fig.1A compares electrophoretic mobilities of ferredoxin-NADP$^+$ oxidoreductase and component polypeptides of isolated cytochrome *b–f* complex, following denaturation in SDS. The flavoprotein (lanes 1,2) appears as a single Coomassie-staining band at 37 kDa; absence of polypeptides of lower M_r attests to the prevention of proteolytic degradation [4] during purification. Lane 3 of fig.1A shows cytochrome *b–f* complex purified as far as the Bio-Gel P300 eluate. In addition to residual contaminating α and β subunits of CF_1 (~55 kDa) the preparation consists of the 5 major polypeptide components belonging to the cytochrome complex, the largest of which co-migrates with ferredoxin-NADP$^+$ oxidoreductase. This component remains with the complex during further purification by sucrose density gradient centrifugation (lane 4) even though there is further loss of CF_1 subunits. As we report below (fig.2) it also remains with the complex through DEAE–cellulose column chromatography, which results in complete removal of CF_1.

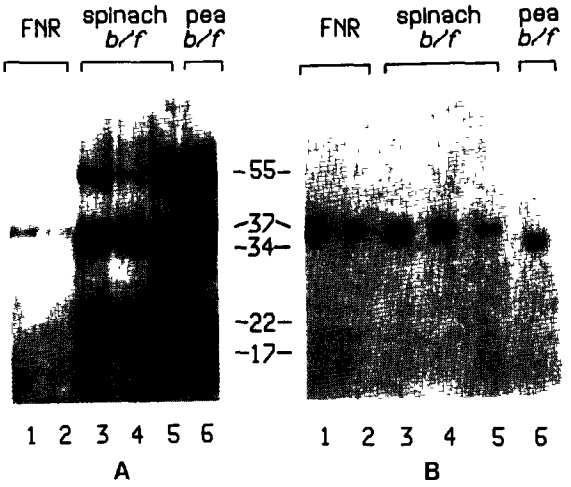

Fig.1. Western blot analysis of ferredoxin-NADP$^+$ oxidoreductase in preparations of cytochrome *b–f* complex from spinach and pea. Samples were run on SDS–PAGE (12% acrylamide, 0.15% bisacrylamide) and stained with Coomassie brilliant blue (A) or transferred electrophoretically to nitrocellulose, then sequentially decorated with antibody to soluble ferredoxin-NADP$^+$ oxidoreductase (FNR) and with ^{125}I-protein A [9] and autoradiographed (B). Samples applied were: (lane 1) 13 pmol soluble enzyme, (lane 2) 6.5 pmol soluble enzyme, (lane 3) 55 pmol spinach cytochrome *b–f* complex, (lane 4) 55 pmol cytochrome complex after sucrose density gradient centrifugation, (lane 5) 55 pmol cytochrome complex after treatment with 2 M LiBr and precipitation from 40% saturated ammonium sulfate [10] and (lane 6) 55 pmol pea cytochrome *b–f* complex.

The 37-kDa component can be selectively removed by treating the isolated cytochrome *b–f* complex with 2 M LiBr (fig.1A, lane 5) or by washing thylakoids with 2 M NaBr prior to extraction of the complex [10]. In cytochrome *b–f* complex prepared from pea thylakoids (lane 6), cytochrome *f* migrates at 37 kDa [11,12] and the polypeptide of 34 kDa does not stain for heme (not shown).

Fig.1B shows the immunoreaction between the components displayed in fig.1A and antibody to ferredoxin-NADP$^+$ oxidoreductase. The relative grain densities appeared at 37 kDa in lanes 1–5 are comparable to the Coomassie stain densities in the corresponding lanes of fig.1A. In particular, treatment of the isolated complex with LiBr (lane 5) results in a proportional loss of staining and grain density in fig.1A and 1B, respectively. The autoradiogram reveals that the material staining at 34 kDa in lane 6 of fig.1A is indeed ferredoxin-NADP$^+$ oxidoreductase [12] and that there is no corresponding component at 37 kDa in pea. Further quantitative analyses were necessary, however, to demonstrate conclusively that the flavoprotein is the only 37-kDa polypeptide in the spinach cytochrome *b–f* complex.

Preparative gel electrophoresis in SDS [13] was performed on cytochrome *b–f* complex from spinach to obtain sufficient 37-kDa polypeptide

Table 1

Amino acid analysis of spinach ferredoxin-NADP$^+$ oxidoreductase and of the 37-kDa component of the cytochrome *b–f* complex

Amino acid	37 kDa	FNR	FNR from [14]
Ala	8.1	6.9	7.1
Arg	4.4	3.2	3.1
Asx	9.2	9.3	9.5
Glx	10.6	11.1	11.6
Gly	10.2	9.0	8.8
His	1.7	1.8	1.7
Ile	4.8	5.0	4.7
Leu	8.2	8.2	8.1
Lys	10.4	10.9	11.7
Met	2.2	4.2	3.7
Phe	4.2	4.4	4.2
Pro	4.8	5.5	5.3
Ser	4.7	4.5	5.1
Thr	4.4	4.6	5.3
Tyr	3.0	4.0	3.7
Val	6.4	7.2	6.3

Amino acids were determined [23] as mol% after 20 h acid hydrolysis; tryptophan and cysteine were excluded from analysis. Ferredoxin-NADP$^+$ oxidoreductase (FNR) was isolated from spinach as described in section 2. Values from [14] are shown for comparison

for amino acid analysis. Table 1 compares the amino acid composition of the 37-kDa polypeptide with that of isolated ferredoxin-NADP$^+$ oxidoreductase and with literature values [14] for this enzyme. Agreement among the analyses is excellent, the only significant deviation being the somewhat lower methionine content of the 37-kDa component, which may reflect partial oxidation during isolation or electrophoresis of the polypeptide.

Partial CNBr cleavage of purified, soluble enzyme and of 37-kDa polypeptide gave essentially identical peptide fragment patterns in the low-M_r range (fig.2). Differences in relative yields of the most slowly migrating fragments are evident and may reflect differences in methionine oxidation noted above or differing rates of cleavage. Cleavage products from cytochrome *f* are shown in lane 5 for comparison.

The above results suggest that ferredoxin-NADP$^+$ oxidoreductase is firmly bound to the

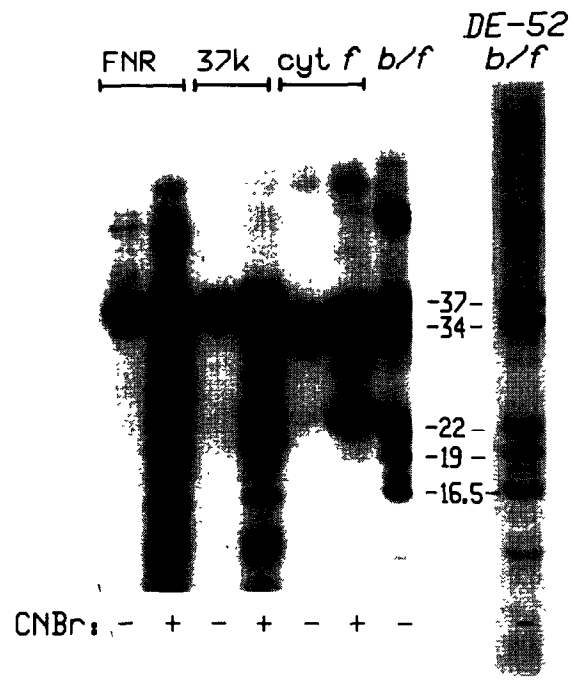

Fig.2. Cyanogen bromide cleavage of soluble ferredoxin-NADP$^+$ oxidoreductase and of polypeptides of the cytochrome *b–f* complex. Samples were incubated with (+) or without (−) a crystal of CNBr in 70% formic acid, 0.1% SDS for 6 h at 0°C, then lyophilized three times before electrophoresis. Samples were: soluble ferredoxin-NADP$^+$ oxidoreductase (FNR), 37-kDa polypeptide from the cytochrome *b–f* complex (37k), cytochrome *f* (cyt *f*) and undissociated cytochrome *b–f* complex at two stages of purification, the Biogel P300 eluate before (*b–f*) and after (DE-52 *b–f*) column chromatography on DEAE–cellulose. The column was equilibrated with 12.5 mM cholate, 30 mM octyl glucoside in 10 mM Na$^+$ morpholinopropanesulfonate (pH 7.2); the cytochrome complex was eluted with 0.4 M KCl in the equilibration buffer and desalted using a Sephadex G-25 column.

cytochrome *b–f* complex. This is confirmed by co-elution of the polypeptides from DEAE–cellulose (fig.2). Furthermore, ferredoxin-NADP$^+$ and PQ-plastocyanin oxidoreductase activities, respectively, characteristic of the flavoprotein and the cytochrome *b–f* complex, co-elute from Bio-Gel P300 in the final step of complex purification (fig.3); ferredoxin-NADP$^+$ oxidoreductase does not appear elsewhere in the elution profile (not shown).

A striking demonstration of the strength of

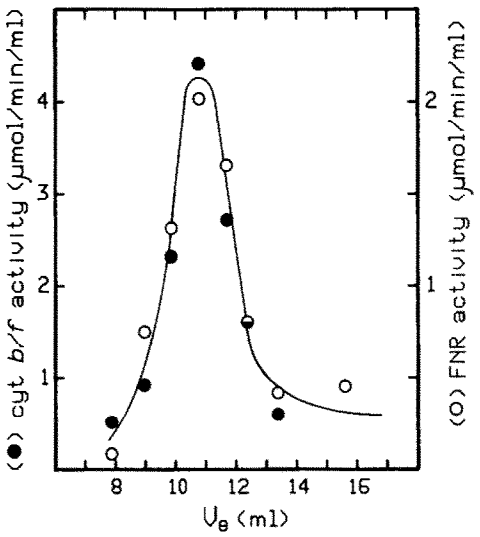

Fig.3. Co-elution of ferredoxin-NADP$^+$ oxidoreductase and cytochrome *b–f* complex from Bio-Gel P300. After fractionation by ammonium sulfate precipitation [1], cytochrome complex was resuspended in 30 mM octyl glucoside, 12 mM sodium cholate, 30 mM Tris–succinate (pH 6.4) and applied to a 15 cm Bio-Gel P300 column equilibrated with 20 mM sodium Tricine, 12 mM sodium cholate (pH 7.5) then eluted with this same buffer. Fractions were assayed for PQ-1:plastocyanin oxidoreductase (●) or diaphorase (○) activity.

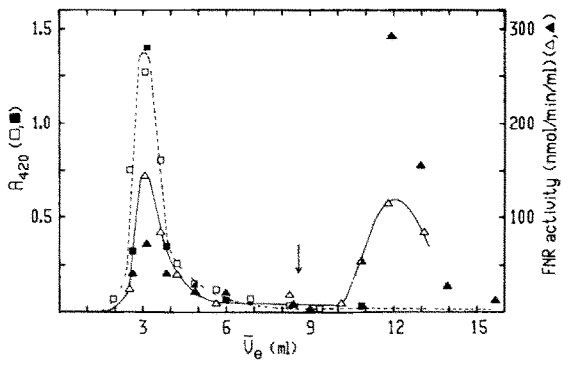

Fig.4. Passage of cytochrome *b–f* complex through phosphocellulose. Spinach cytochrome complex was diluted to 2.4 mM sodium cholate, 3 μM cytochrome *f* in 20 mM sodium Tricine (pH 7.5) and passed through phosphocellulose preequilibrated with Tricine–cholate buffer. Adsorbed ferredoxin-NADP$^+$ oxidoreductase (FNR) was eluted with 0.2 M potassium phosphate buffer containing 2.4 mM sodium cholate (arrow). Cytochrome content (A_{420}; □, ■) and diaphorase activity (△, ▲) were determined for complex chromatographed before (open symbols) or after (solid symbols) treatment with 2 M LiBr (1 h at 0°C) and desalting by centrifugation through Sephadex G-25. Elution volumes have been normalized ($\bar{V}_e$) to allow for differences in scale of the two experiments, each of which was run at a sample:bed volume ratio of 1:2.8.

association between the flavoprotein and the complex was obtained using chromatography over phosphocellulose (fig.4), which is known to bind free ferredoxin-NADP$^+$ oxidoreductase [3]. Neither cytochrome *b–f* complex nor the majority of diaphorase activity was significantly retarded during passage through phosphocellulose, though a small amount of what appeared to be free flavoprotein (present originally or released upon exposure to phosphocellulose) was retained by the column and could be subsequently eluted at high ionic strength. When cytochrome *b–f* complex that had been treated with 2 M LiBr and then desalted was applied to the phosphocellulose column, less diaphorase activity eluted with cytochrome and more was retained by the phosphocellulose (fig.4). A marked increase in diaphorase specific activity was evident upon dissociation of the ferredoxin-NADP$^+$ oxidoreductase from the complex. A similar increase was also evident when cytochrome *b–f* complex was exposed to LiBr and not chromatographically fractionated before assay; no increase was observed upon treatment of the isolated enzyme with 2 M LiBr (not shown).

4. DISCUSSION

A fraction of the reductase is tightly bound to the cytochrome *b–f* complex, as shown by co-purification through ammonium sulfate precipitation, gel filtration on Bio-Gel P300, density gradient centrifugation, DEAE–cellulose chromatography and phosphocellulose chromatography. Binding of enzyme to thylakoids or to isolated cytochrome *b–f* complex is clearly weakened, however, by treatment with chaotropic salt (fig.1,4; [10]) which probably accounts for its absence from other preparations of cytochrome *b–f* complex [2,15,16]. Furthermore, prolonged fractionation of cytochrome *b–f* complex, particularly in systems that physically separate pro-

teins on the basis of molecular mass, may be expected to leach the enzyme from the complex by pulling the binding equilibrium [17] in the direction of dissociation; partial release of the 37-kDa protein on 20 h sucrose gradient runs has already been reported [1].

Demonstration that the 37-kDa polypeptide is ferredoxin:$NADP^+$ reductase raises the question: Should this enzyme be considered a component of the cytochrome *b–f* complex? Although it does not qualify as a core (i.e., membrane-spanning) component of the cytochrome complex, we believe that it should be regarded as a peripheral component for the following reasons:

(i) Several estimates [24–26] indicate that over 80% of the chloroplast ferredoxin:$NADP^+$ reductase is bound to the thylakoids, where it occupies a single class of binding site [18]. Co-purification of reductase with the cytochrome complex ([1], fig.3) strongly supports the concept that the cytochrome complex provides this site. Analysis of washed thylakoids by Western transfer [9] indicates that ~2 nmol tightly bound flavoprotein are retained per μmol chlorophyll (in preparation), in reasonable agreement with the results of kinetic analyses [18] and corresponding to attachment of ~1 molecule of enzyme to each cytochrome complex, of which thylakoids also contain ~2 nmol/μmol chlorophyll [1,2,19,20]. (Inclusion of ferredoxin-$NADP^+$ oxidoreductase in estimating the mass of cytochrome *b–f* complex in situ yields a sum of ~135 kDa if the complex is made up of a single copy of each polypeptide; this value is in good agreement with the notion that the cytochrome *b–f* complex, when reconstituted into liposomes, is a dimer of ~280 kDa [15].)

(ii) A distinction may be drawn between electron carriers which are components of the cytochrome complex and those soluble proteins such as ferredoxin and plastocyanin which are, in a sense, its substrates and probably function as mobile electron shuttles. Both of these latter are largely depleted when thylakoids are ruptured and are completely removed upon detergent extraction, as judged by SDS gel electrophoresis and analysis by EPR spectroscopy (not shown). In contrast, the majority of the reductase is found associated with the cytochrome complex, even after solubilization of the thylakoids with non-ionic detergents [1].

(iii) Comparison between the binding of reductase to the cytochrome complex and that of CF_1 to its transmembrane complement, CF_0, is instructive, since CF_1 is an acknowledged component of the coupling factor complex. Both reductase and CF_1 are linked to their respective binding site through ionic forces involving a divalent cation [18,27], but the latter is less tightly bound and can be washed away at low ionic strength to expose the reductase (which it shields) and permit agglutination of thylakoids by anti-reductase antiserum [28,29]. Both reductase and CF_1 possess enzyme activities that are independent of participation by the membrane-bound component, but in both cases, binding to the membrane extends and modifies these activities. For example: (i) when reductase binds to thylakoids its FAD domain becomes sensitive to the internal thylakoid pH [30] (cf. CF_1 binding to CF_0 [27]); (ii) chemical modifications of bound reductase [31] and bound CF_1 [27] are sensitive to illumination; (iii) diaphorase activity of the reductase toward indophenol dye decreases when the enzyme is released from thylakoids [18], while ferredoxin-dependent cytochrome *c* reduction increases when the enzyme is released from isolated cytochrome complex (fig.4); ATPase activity is elicited more readily after release of CF_1 from the membrane [27].

What is the physiological significance of the attachment of FNR to the cytochrome *b–f* complex? Electron flow from NADPH to plastoquinone [32] and cytochrome b_6 [33] is demonstrable in thylakoids and is presumed on the basis of inhibitor sensitivities and ferredoxin requirement to involve reductase. In a modified Q-cycle scheme proposed previously [21] it was suggested that efficient reoxidation of cytochrome *b*-563 during cyclic electron flow requires the contribution of an electron from reduced ferredoxin-$NADP^+$ oxidoreductase to accomplish complete reduction of a plastoquinone molecule. A binding site for the enzyme on the cytochrome complex was also postulated [22] on the basis of inhibitor studies which suggested its involvement in cyclic electron flow around Photosystem I. Such a physical location could permit the reductase to regulate the distribution of electrons between the linear and cyclic pathways [22] by directing them into the stroma or returning them to the membrane, respectively.

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