The Ferredoxin-NADP+ Oxidoreductase-binding Protein Is Not the 17-kDa Component of the Cytochrome b/f Complex*

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The complex between ferredoxin-NADP+ oxidoreductase and its proposed membrane-binding protein (Vallejos, R. H., Ceccarelli, E., and Chan, R. (1984) J. Biol. Chem. 259, 8048–8051) was isolated from spinach thylakoids and compared with isolated cytochrome b/f complex containing associated ferredoxin NADP+ oxidoreductase (Clark, R. D., and Hind, G. (1983) J. Biol. Chem. 258, 10348–10354). There was no immunological cross-reactivity between the 17.5-kDa binding protein and an antiserum raised against the 17-kDa polypeptide of the cytochrome complex.

Association of ferredoxin-NADP+ oxidoreductase with the binding protein or with the thylakoid membrane gave an allotopic shift in the pH profile of diaphorase activity, as compared to the free enzyme. This effect was not seen in enzyme associated with the cytochrome b/f complex.

Identification of the 17.5-kDa binding protein as the 17-kDa component of the cytochrome b/f complex is ruled out by these results.

Chloroplast ferredoxin-NADP+ oxidoreductase catalyzes the final step in linear electron transport: the reduction of NADP+. In preparations of thylakoid membranes, weakly and tightly bound pools of the enzyme can be detected (1). The tightly bound pool is subject to allotopic effects which distinguish it from weakly bound and free enzyme (2). Involvement of the bound enzyme in light-dependent modulation of both linear (2) and cyclic (3) electron transport has been proposed.

The membrane-bound reductase is nevertheless an extrinsic protein which can be detached from the membrane by washing with low-salt buffer or EDTA (4) or by mild trypsinization of the membrane (5). Carrillo and Vallejos (4), on the basis of rebinding studies, suggested that there was a specific binding site on the membrane for the reductase; they subsequently isolated a complex between the enzyme and a 17.5-kDa protein that showed an allotopic effect characteristic of the membrane-bound enzyme (6). Independently, Clark and Hind (7) isolated a cytochrome b/f complex containing an additional 37-kDa polypeptide that was subsequently identified as ferredoxin-NADP+ oxidoreductase (8). Since the cytochrome b/f complex also contains a ~17-kDa subunit (9), it seemed possible that this subunit could be the 17.5-kDa binding protein of Vallejos et al. (6).

Identity between these polypeptides and their candidacy as the membrane-binding site for the reductase in vivo is examined by affinity chromatography and by immunological and enzymological approaches.

MATERIALS AND METHODS

Ferredoxin-NADP+ oxidoreductase was isolated from spinach thylakoids by affinity chromatography on Cibacron Blue (10). The five-polypeptide cytochrome b/f complex was isolated as in Ref. 7, with omission of the cytochrome-c-Sepharose column.

Diaphorase activity was measured by monitoring the reduction of 0.5 mM potassium ferricyanide at 420 nm in 50 mM Tris buffer (pH 9.0), using 200 μM NADPH as electron donor (11). SDS-gel electrophoresis was performed as in Ref. 12, using the gel system described by Studier (13). Fifteen percent (w/v) acrylamide, 0.2% (w/v) N,N'-methylene bisacrylamide gels were routinely used for separation and identification of polypeptides, but 12% (w/v) acrylamide, 0.16% (w/v) N,N'-methylene bisacrylamide gels were used when subsequent electrophoretic transfer of polypeptides to nitrocellulose (14) was intended; this was carried out for 1 h at 60 V, then 1 h at 100 V in a Hoefer Transphor TE 50. Immunological detection of the 17-kDa component of the cytochrome b/f complex and of ferredoxin-NADP+ oxidoreductase on the nitrocellulose sheet was performed as in Ref. 15, using rabbit antisera to the individual proteins and 125I-labeled protein A. Gels were fixed and silver-stained as in Merrill et al. (16). Antisera to the reductase and the 17-kDa component of the cytochrome b/f complex were raised as in Ref. 17.

RESULTS AND DISCUSSION

Vallejos et al. (6) used affinity chromatography on a Cibacron Blue-Sepharose column to purify the ferredoxin-NADP+ oxidoreductase-binding protein complex. Fig. 1 presents a comparison of the elution profiles of the free reductase and reductase complexed to either the binding protein or the cytochrome b/f complex. As observed by Vallejos et al. (6), the peak of diaphorase activity was associated with the reductase-binding protein complex eluting in ~150 mM NaCl. In contrast, both free reductase and reductase initially associated with the cytochrome b/f complex eluted in 300 mM NaCl. The cytochrome b/f complex itself remained bound to the column and could be eluted by washing the Cibacron Blue-Sepharose with an ionic detergent (10 mM CHAPS* or cholate).

Immune blotting (Fig. 2A) showed no cross-reactivity between the binding protein and an antiserum raised against the isolated 17-kDa polypeptide. Furthermore, direct comparison between the binding protein and the 17-kDa component of the cytochrome b/f complex by sodium dodecyl sulfate gel electrophoresis revealed a difference in the electrophoretic mobility of the two polypeptides (Fig. 2B) and in the color of the silver stain (not shown).

An allotopic effect of membrane-binding upon ferredoxin-NADP+ oxidoreductase is readily discerned as an acid shift

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1The abbreviations used are: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; Hepes, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; Mes, 3-(N-morpholino)ethanesulfonic acid; Tricine, N-tris(hydroxymethyl)methylglycine.

14891
Identity of NADP⁺-reductase Binding Protein in Thylakoids

Fig. 1. Affinity chromatography of ferredoxin NADP⁺oxidoreductase on Cibacron Blue. Twenty units (6) of diaphorase activity in a 5% (v/v) Triton X-100 thylakoid extract (6), or in cytochrome b/f complex prepared as in Ref. 7 with addition of Triton X-100 to 0.5% (v/v), or as purified reductase with the addition of Triton X-100 to give 1.0% (v/v) were loaded on a 1-ml (6 × 0.5-cm) Cibacron Blue-Sepharose column preequilibrated with 0.1% (v/v) Triton X-100, 1 mM MgCl₂, 10 mM Tricine/NaOH (pH 8.0). The column was rinsed with 10 bed volumes of buffer and eluted with 20 ml of a linear 0-0.5 M NaCl gradient. Fractions (~600 μl) were assayed for diaphorase activity, protein content, and polypeptide composition. ○—○, Triton X-100 extract; ■—■, reductase and cytochrome b/f complex; □—□, reductase alone.

Fig. 2. A comparison of the immune cross-reactivities and electrophoretic mobilities of the reductase binding protein and the 17-kDa polypeptide of the cytochrome b/f complex. A, immune blot. Lane 1, cytochrome b/f complex prepared as in Ref. 7. Lane 2, cytochrome b/f complex further purified on a sucrose gradient as in Ref. 9. Lanes 3 and 4 are two different preparations of the reductase-binding protein complex. The nitrocellulose blot was probed with a mixture of rabbit anti-reductase and anti-17-kDa polypeptide (of the cytochrome b/f complex). B, silver stain. Lanes 1–4 are the same samples as in the immune blot.

in the pH profile of diaphorase activity (4). This property was used to characterize the different preparations containing reductase (Fig. 3). Both membrane-bound ferredoxin-NADP⁺oxidoreductase and the reductase-binding protein complex show a typical acid shift of ~1.0 pH unit, with respect to the free enzyme, in the pH profile of diaphorase activity. In contrast, the activity profile of enzyme associated with cytochrome b/f complex is almost identical to that of free reductase.

The present investigation was designed to test the hypothesis that the ferredoxin-NADP⁺ reductase-binding protein isolated by Vallejos et al. (6) is identical to the 17-kDa subunit of the cytochrome b/f complex (7, 9). This was found not to be the case, as the two proteins differ in electrophoretic mobility, silver staining, and immune reactivity. Furthermore, reductase present in the cytochrome b/f complex does not show the allotropic, acid pH shift in diaphorase activity that is characteristic of the membrane-bound enzyme and of enzyme associated with the 17.5-kDa binding protein.

The significance of the association between reductase and the cytochrome b/f complex is called into question by these findings, for it can be argued that such a loose association arises as an artifact of the isolation procedure. It is noteworthy, however, that increased activity of both ferredoxin-dependent cytochrome c reduction and diaphorase is observed when reductase is removed from the cytochrome b/f complex either by treatment with LiBr (8) or by density gradient centrifugation. This effect was noted earlier by Fredricks and Gehl (18), upon release of reductase from thylakoid membranes. Unpublished data further reveal that there are two pools of reductase associated with thylakoids, one weakly and one tightly bound. The possible existence of two classes of binding site is now being examined by covalently cross-linking the reductase to thylakoid membranes.

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