Removal of Ferredoxin:NADP\(^+\) Oxidoreductase from Thylakoid Membranes, Rebinding to Depleted Membranes, and Identification of the Binding Site* 

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Ferredoxin-NADP\(^+\) oxidoreductase associates with thylakoid membranes into two pools of different binding strength that are experimentally distinguished on the basis of resistance to removal by washes in low ionic strength media. The non-denaturing zwitterionic detergent 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid is uniquely able to remove the more tightly bound pool of enzyme, without solubilization of major membrane proteins. The reconstitution of reductase onto depleted thylakoid membranes requires available membrane binding sites and cations, in order of effectiveness trivalent > divalent > monovalent. The heterobifunctional \(^125\)I-iodinated Denayer-Jaffe cross-linking reagent yields a 54-kDa, covalently cross-linked adduct between ferredoxin-NADP\(^+\) oxidoreductase and a component of the thylakoid membrane. Our results show that the more tightly bound pool of enzyme is associated with the 17.5-kDa reductase-binding protein (Vallejos, R. H., Ceccarelli, E., and Chan, R. (1984) *J. Biol. Chem.* 259, 8048–8051).

The reduction of NADP\(^+\) by ferredoxin:NADP\(^+\) oxidoreductase is the terminal step in the electron transport chain of the thylakoid, and the point at which reductant is delivered to the stromal compartment. The observation of Shin et al. (1) that a significant amount of this enzyme is bound to the thylakoid membrane is frequently overlooked. Recent evidence of its sensitivity to thylakoid energization (2, 3) has, however, strengthened the view (4, 5) that the reductase is functionally associated with the membrane. A more tightly bound pool is alleged to comprise between 30 and 60% of the total enzyme (6–10), as determined from the release of diaphorase activity in various wash treatments. The variability in these estimates may, in part, be due to an allotropic effect on the more tightly bound pool of enzyme (11–13) that influences its specific activity as a diaphorase.

Crude localization of thylakoid-bound reductase\(^1\) by immuno-reactivity suggested its association with stacked and unstacked membranes (11) and screening of its antigenic sites by coupling factor 1 (14). More recently, reductase was found to co-purify with the cytochrome b/f complex (15) and was postulated to associate with it in situ. However, a \(\approx17.5\)-kDa thylakoid protein which, after isolation from solubilized membranes, binds reductase (16) and exerts an allotropic effect thereon, is a more likely candidate for the tight binding site (9, 16–18). The orientation of this 17.5-kDa protein in the membrane and its relation to the major protein complexes are unknown; its actual functioning as an in situ binding site for the reductase is unsubstantiated.

Identification or further characterization of reductase binding sites has awaited development of a methodology for completely removing enzyme from the membranes and then reconstituting with native or labeled enzyme. Rebinding studies have been performed on membranes depleted by washing with EDTA (7), by washing with low ionic strength media containing CaCl\(_2\) (3), or by trypsin treatment (9), but interpretation of these results may be questioned since the membranes evidently retained some tightly bound enzyme.

This communication presents a procedure, using the zwitterionic detergent CHAPS (19), for discriminating between the loosely and tightly bound pools of reductase and for solubilizing almost the entire reductase pool from thylakoids. Conditions for rebinding reductase to depleted membranes are described. A heterobifunctional, photoactivable, cross-linking reagent has been employed as a probe for reductase binding sites and has demonstrated the in situ functioning of a binding protein of \(\approx17\) kDa.

**MATERIALS AND METHODS**

**Thylakoid Preparation and Treatment—**Spinach (*Spinacia oleracea*, cv. Park Hybrid 424) was grown under a combination of fluorescent (5160 watts) and incandescent (300 watts) lights at an incident intensity of 60 watts/m\(^2\). The growth chamber was maintained on a 10-h (20°C)/14-h (16°C) light/dark cycle. Leaves were collected from 6- to 10-week-old plants. All preparation procedures were done at 0–4°C.

Intact chloroplasts were obtained by the method of Walker (20). A chloroplast sample (300–500 \(\mu\)l, equivalent to 1 mg of chlorophyll) was brought to 1 ml with either 5 mM NaCl, 10 mM sodium Tricine (pH 7.8), or with 1 mM EDTA (pH 7.0). After 10 min on ice, thylakoids were sedimented in an Eppendorf centrifuge (12,000 \(\times\) g, 30 s; defined as Wash 1), and the supernatants were decanted. Additional washes in the appropriate buffer were performed in the same way. Thylakoids were finally resuspended in 1 ml of the buffered 5 mM NaCl or were treated as follows.

Washed thylakoids (1 \(\mu\)mol of chlorophyll) were suspended in 1 ml of 10 mM CHAPS, 10 mM MgCl\(_2\), 10 mM sodium Mops (pH 7.2) and stored on ice for 10 min. Sedimentation (12,000 \(\times\) g, 10 min) yielded a clear yellow supernatant containing reductase. The extracted pellet was resuspended in buffered 5 mM NaCl. In some experiments (as indicated), the pelleted, intact chloroplasts were directly resuspended...
in CHAPS medium and washed 3 times with it, before final resuspension in 5 mM NaCl, 10 mM sodium Tricine (pH 7.8).  

Reconstitution—Depleted membranes (1 μmol of chlorophyll/ml) were supplemented with purified enzyme (21) equivalent in diaphorase activity (measured as below) to that of native membranes. Cations were present as indicated, and the samples were incubated, with gentle agitation, for 20 min at 20 °C. After centrifugation (12,000 × g, 10 min), diaphorase activity was measured in the pellet and supernatant. The membranes were washed three times in 5 mM NaCl, 10 mM sodium Tricine (pH 7.8) and assayed as before.  

Identification of Enzyme Binding Site—Cross-linking between reductase and the membrane was achieved using the Denny-Jaffe reagent (22, 23). Enzyme (5 nmoI in 0.1 ml of 0.1 M NaHCO₃ (pH 8.5)) was added to 10 pmol (10 μCi) of 32P-labeled Denny-Jaffe reagent (from 5 μl of benzene, blown to dryness with dry N₂) and incubated for 1 h in the dark at 20 °C. The reaction was quenched by the addition of 10 μl of 1 M Tris chloride (pH 8.0), and the products were fractionated by passage through a 2-ml column (3 × 0.5 cm) of Sephadex G-25, equilibrated with 0.1 M NaHCO₃ (pH 8.5). Fractions containing enzyme were pooled and stored in darkness at 0 °C. Incorporation of 40–50% of the radiolabel was obtained. Aliquots of resolved thylakoid membranes (as indicated) were pipetted into glass reaction vials containing 2.5 μl of 0.5 mM potassium ferricyanide, 50 μl of CHAPS (2.0-4-chlorobenzenephyl)-1,1-dimethylurea (to inhibit photosynthetic electron transport and oxidize endogenous reductants). Derivatized enzyme (50 μl, ~0.5 μCi) was added, and the mixture was incubated for 10 min at 20 °C in darkness, for rebinding to occur. Samples were then irradiated with blue light (500-watt tungsten source; Corning filter CF 4-76, 1000 watts·m⁻²) for 5 min at 0 °C. The membranes were precipitated by centrifugation (12,000 × g, 3 min) and washed in 0.1 M NaHCO₃ until the supernatant radioactivity reached background. The pellets were solubilized as described below and subjected to electrophoresis on a 15% acrylamide, 0.2% bisacrylamide gel. The gel was stained with Coomassie Brilliant Blue, destained, and then dried for autoradiography at -70 °C using Cronex x-ray film and an intensifying screen. The distribution of radioactivity was quantified by cutting out the radioactive areas of the dried gel and counting the sections in a Beckman γ-ray counter.  

Assay Procedures—Diaphorase activity was measured at 20 °C in a Cary 14 recording spectrophotometer. Reference and measuring cuvettes contained 0.5 ml of 20 mM sodium Taps (pH 8.9), 1 mM potassium ferricyanide, and 10 μl of the thylakoid or supernatant sample. The reaction was started by adding 10 μl of 10 mM NADPH to the measuring cuvette. Ferricyanide reduction was monitored at 420 nm. At the pH used, solubilized and bound reductases show the same activity on a protein basis; that is, there is no allotopic effect (11, 12).

Photosynthetic electron transport to ferricyanide was monitored in an O₂ electrode; samples contained 10 mM sodium Tricine, 5 mM MgCl₂, 1 mM potassium ferricyanide, and 25 μM chlorophyll. Chloroplast inclusions were estimated by a standard procedure (24). Plastocyanin (5 μM) and NH₄Cl (5 mM) were added as indicated.  

Gel electrophoresis followed a standard method (25) using 15% acrylamide, 0.2% bisacrylamide gels. Samples were dissolved in 4% sodium dodecyl sulfate, 4% β-mercaptoethanol, 25% glycerol, 25 mM Tris chloride (pH 8.0), and the gels were stained with Coomassie Brilliant Blue R-250. The molecular mass standards were: phosphorylase b (92.5 kDa), bovine serum albumin (67.0 kDa), egg albumin (45.0 kDa), chymotrypsinogen (25.0 kDa), myoglobin (17.8 kDa), and cytochrome c (12.3 kDa). Protein content was determined by the Bradford procedure (26) and chlorophyll, by the method of Arnon (27).  

Chemicals—Denny-Jaffe reagent was supplied by New England Nuclear Research Products. All other reagents were from Sigma.

RESULTS  

The release of ferredoxin: NADP⁺ oxidoreductase from chloroplasts (>70% intact) subjected to osmotic shock in low ionic strength medium and from the repeatedly washed thylakoids is shown in Fig. 1. Washes in 5 mM NaCl (or 5 mM MgCl₂, not shown) removed ~60% of the total reductase content (defined as the loosely bound pool). Extensive washing in 1 mM EDTA released some of the remaining bound enzyme, but only the detergent CHAPS was effective in substantially removing this tightly bound pool. The importance of using more than the critical micellar concentration (~6 mM) of CHAPS is demonstrated in Fig. 2A. Fig. 2B shows that the effect of a single exposure to CHAPS is >90% complete within 5 min. Removal of almost all reductase from the chloroplasts was obtained by washing three times in buffered 10 mM CHAPS (Fig. 1).  

Fig. 3 shows the pattern of protein release obtained by exposure of washed thylakoids to 10 mM CHAPS. The prominent 37-kDa protein was identified as reductase on the basis of its reaction with antiserum raised against the purified enzyme (Western blot not shown) and correlation of its staining intensity with the diaphorase activity of the sample before electrophoresis. The release of reductase by CHAPS was paralleled by loss of a 10-kDa protein identified as plastocyanin (Western blot not shown). A 32-kDa component that did not cross-react with antiserum to reductase (which frequently gives proteolysis products in this mass range) may be a peripheral PS 2 protein (28, 29). The 12-, 21-, and 23-kDa polypeptides and components of higher molecular mass were not identified.  

This CHAPS washing procedure is remarkably selective, in
that coupling factor 1 (and possibly other peripheral proteins) remains bound to the membranes. The oxygen-evolving complex is relatively insensitive to CHAPS, for, although photo-extraction with CHAPS. Membrane fractions containing 1 μmol of chlorophyll·ml⁻¹ (8 μl) and CHAPS supernatant (30 μl) were mixed with double their volume of solubilization buffer and electrophoresed. Lane 1, thylakoid membranes washed five times with NaCl; lane 2, CHAPS extract of lane 1; lane 3, membranes after CHAPS extraction. FNR, ferredoxin:NADP⁺ oxidoreductase; PC, plastocyanin.

**TABLE I**

| Washing agent | Residual reductase | Added with reductase | Reductase rebound |
|---------------|-------------------|---------------------|------------------|
| NaCl          | 1.53              | 1.53 NaCl, 125 mM   | 1.80             |
| NaCl          | 1.53 MgCl₂, 10 mM | 1.63                 | 0.97             |
| EDTA          | 1.03              | 1.03 NaCl, 125 mM   | 1.63             |
| EDTA          | 1.03 MgCl₂, 10 mM | 1.61                 | 0.97             |
| NaCl, CHAPS   | 0.32              | 0.32 NaCl, 125 mM   | 0.58             |
| NaCl, CHAPS   | 0.32 MgCl₂, 10 mM | 1.12                 | 0.60             |

*Includes residual reductase.

**TABLE II**

**Rebinding of reductase to thylakoid membranes**

Thylakoids were resolved by five washes with NaCl, followed by a CHAPS wash as indicated, or by five washes with EDTA. After incubating with 3 nmol of enzyme/μmol of chlorophyll, the membranes were washed three times in resuspending buffer. Data shown are nmol/μmol of chlorophyll ± 10% S.D. Assays on the supernatant fractions gave confirmatory results (not shown). Similar data were obtained with 1 mM dithioerythritol present throughout.

**Cation requirement for rebinding of reductase**

Thylakoids were washed three times in CHAPS, as for Fig. 1, giving a residual reductase content of 0.60 nmol/μmol of chlorophyll. Reductase (4 nmol) was reconstituted as described under "Materials and Methods," in the presence of the salts shown. Values are nmol of tightly bound enzyme/μmol of chlorophyll.

**TABLE III**

| Added salt | KCl | NaCl | CaCl₂ | MnCl₂ | MgCl₂ | LaCl₃ |
|------------|-----|------|-------|-------|-------|-------|
| Concentration (mM) | 125 | 125 | 10 | 10 | 10 | 3 |
| Reconstituted enzyme | 0.54 | 0.38 | 0.38 | 0.63 | 0.80 | 1.57 |

**DISCUSSION**

Our results agree with earlier reports (10, 11) in showing that reductase associates with thylakoid membranes into a weakly bound and a tightly bound pool. Intact chloroplasts have been found to contain 4 nmol of reductase/μmol of chlorophyll (9-11, this work). Approximately half of this is readily removed by osmotic shock. Thereafter, repeated washings in either buffered NaCl or MgCl₂ leave 1.6 nmol/μmol of chlorophyll bound to the membranes (Fig. 1). This pool (40%) is defined as the tightly bound reductase. It may be partially removed by washing in EDTA, or in 5 μM CaCl₂, or by trypsin digestion (3, 6-8); these procedures leave on the membrane 0.7-1.2 nmol of enzyme/μmol of chlorophyll. In contrast, washing with CHAPS removes the bound reductase with a high degree of specificity (Fig. 3), leaving a bound residue of only 0.1-0.4 nmol/μmol of chlorophyll.

Rebinding of up to 1.5 nmol of reductase/μmol of chlorophyll could be achieved by use of CHAPS-washed membranes. This rebinding was dependent on cations, the order of effectiveness being trivalent > divalent > monovalent. A small amount of rebinding to EDTA-treated thylakoids was observed, but scarcely any binding could be detected by control membranes containing the full complement of bound reductase (Table II). We conclude, therefore, that binding of reductase to thylakoid membranes is possible only after partial removal of the enzyme with EDTA or similar treatments, or
FIG. 4. Autoradiograph of [35S]-iodinated reductase, reconstituted and covalently cross-linked to washed thylakoids. Membranes were washed with either CHAPS or EDTA aad reconstituted as described in the legend to Table II. Radiolabeled enzyme was prepared as described under "Materials and Methods." A., lanes 1, 2, and 3, reconstitution to CHAPS-washed membranes in the presence of MgCl₂ or NaCl or without salt, respectively. Lanes 4 and 5, reconstitution to EDTA-washed membranes with or without MgCl₂, respectively. Lane 6, as lane 1, but omitting the second (light-dependent) cross-linking step. Lane 7, radiolabeled enzyme without membranes. B, Coomassie Blue stain of the enzyme preparation used for cross-linking.

its essentially complete removal by CHAPS.

Fig. 4 supplies evidence in support of the claim (16, 18) that the binding site for reductase (37 kDa) on the thylakoid membrane is comprised of a polypeptide of ≈17.5-kDa apparent molecular mass. Appearance of a 54-kDa adduct (37 + 17 = 54) depended on prior washing of the membranes in CHAPS; EDTA treatment was ineffective in exposing the relevant binding sites. Since only CHAPS removes the major portion of tightly bound reductase from the membrane, as discussed above, it seems reasonable to conclude that the 17.5-kDa binding protein provides the sites to which reductase is tightly bound.

Shin and co-workers (31, 32), on the other hand, have claimed that a 10-kDa protein, termed "connectein," is directly responsible for linking reductase to an intrinsic membrane attachment site. Such a linker protein would not be detected in the experiments reported here, in view of its alleged failure to stain with Coomassie Blue and the presence in our extracts of normally staining plastocyanin with identical molecular mass. The treatments used by Nozaki et al. (32) to deplete thylakoids of reductase did not remove the tightly bound pool, thus, we cannot readily relate our observations to their work. Carrillo and Vallejos (7), however, in a comparable study, did not discern any requirement other than divalent cation for rebinding.

Enzyme covalently modified with Denny-Jaffe reagent is effectively a monovalent probe, given the very low cross-linking efficiency in the photochemical step; thus, our data neither support nor exclude the suggestion (16, 18) that a single molecule of enzyme binds to a trimer of 17.5-kDa binding protein. The binding protein is distinct from the 17.5-kDa polypeptide component of the cytochrome b/f complex (17, 18), and its relation to the major membrane protein complexes is unknown.

Reconstitution of reductase to the 17.5-kDa protein also required ≈10 mM magnesium ion, although 125 mM NaCl was weakly effective (Fig. 4). Carrillo and Vallejos (7) demonstrated the reconstitution of diaphorase activity to EDTA-washed membranes, but no obvious ion specificity was documented; moreover, as shown in Table II, enzyme reconstituted to EDTA-washed membranes may be largely removed again by washing with buffered 5 mM NaCl. It is questionable, therefore, whether complete dissociation from, and reassociation with, the tight binding sites was achieved in that study (7), although rebinding under their conditions led, nevertheless, to the allotopic effect in the pH profile of diaphorase activity.

A requirement for magnesium ion in rebinding reductase to the strong binding sites (Fig. 4), but the resistance of this association to EDTA washes (Table II) indicates that forces other than ionic charge interactions are involved. Hydrophobic interactions could provide additional binding strength and account for sensitivity of the enzyme-membrane association to CHAPS. When the three-dimensional crystal structure of reductase (33) has been refined to higher resolution and related to the amino acid sequence (34), it should be possible to identify surface domains that are potential candidates for hydrophobic and ionic interactions with the binding protein.

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