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Reconstitution of isolated Rieske Fe-S protein into a Rieske-depleted cytochrome b_6-f complex

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The Rieske Fe-S protein can be isolated from the cytochrome b_6-f complex by means of chromatography on a hydroxyapatite column in the presence of detergent. Depletion of the cytochrome complex from the Rieske protein results in the loss of oxidoreductase activity, as well as the ability to reduce cytochrome b_6. The Rieske Fe-S protein can be reconstituted into the Rieske-depleted complex by removal of the Triton X-100 molecules associated with the protein fractions, and their substitution by lipids. Upon reconstitution the complex is reactivated, and the role of the Rieske Fe-S protein in the reduction of both plastocyanin and cytochrome b_6 can be demonstrated.

Cytochrome b_6-f; Rieske Fe-S protein; Reconstitution

1. INTRODUCTION

The cytochrome b_6-f complex is a thylakoid membrane protein complex active as a plastoquinol-plastocyanin oxidoreductase, catalyzing the electron transfer from two- to one-electron acceptors [1]. It is composed of four subunits: cytochrome f, cytochrome b_6, Rieske Fe-S protein and subunit IV. Coupled to this transfer of electrons, the complex also translocates protons across the thylakoid membrane into the lumen. The mechanism of action of the cytochrome b_6-f complex, as well as of a related complex, the cytochrome b-c_1 complex, which is present in mitochondria and bacteria [1], is not yet understood. Two features are common to all cytochrome b-c complexes: two protons are translocated across the membrane as one electron passes through the complex, and the addition of an oxidant induces the reduction of cytochrome b. Two mechanisms have been proposed to explain these common features: the Q-cycle [2] and the b-cycle [3], although support for either one has not yet been exclusively demonstrated.

Several approaches have been used in order to gain a better understanding of the mechanism of action of the cytochrome b_6-f and related complexes. These include studies of electron transport with the isolated complex [4–6], studies of electron transport and proton translocation in a complex incorporated into liposomes [7,8], determination of amino acid sequence and modeling of the complex structure [9–12], and depletion of the complex of essential components and attempts to reconstitute them into the complex [13,14]. In relation to depletion and reconstitution, the complex has been depleted of PQ and lipids to yield an inactive complex [13]. This depleted complex could be reactivated upon addition of both PQ and different lipids, with phosphatidylcholine being the most effective [13]. Depletion of the complex from one of its protein subunits could also be achieved.

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Abbreviations: BCA, bicinchoninic acid; CMC, critical micellar concentration; Mes, 2-(*N*-morpholino)ethanesulfonic acid

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The Rieske protein was resolved from the complex [14], and the remaining Rieske-depleted complex was inactive in both plastocyanin [14] and cytochrome b_6 reduction [5,15]. However, electron transport activity could not be restored by adding the isolated Rieske protein fraction to the depleted complex [14]. Here, we demonstrate the reconstitution of the isolated Rieske protein into the depleted complex with a restoration of electron transport activity. The Rieske Fe-S center is also shown to be obligately required for cytochrome b_6 reduction.

2. MATERIALS AND METHODS

The cytochrome b_6-f complex from spinach leaves was prepared by detergent solubilization and sucrose density gradient centrifugation as described in [4] with few modifications [8]. The complex was resolved into the Rieske Fe-S protein and a Rieske-depleted complex essentially by the method of Hurt et al. [14]: the complex was loaded onto a hydroxyapatite column and eluted first with 20 mM potassium phosphate (pH 6.8) plus 0.5% Triton X-100 and then with 200 mM potassium phosphate (pH 6.8) plus 0.05% Triton. The Rieske protein came off the column in the first elution while the depleted complex was recovered in the second. Further steps of purification of the Rieske protein were eliminated as will be described later.

Reconstitution of the Rieske protein into the Rieske-depleted complex was carried out by replacing the Triton with lipids. Soybean phosphatidylcholine (10 mg), dissolved in chloroform, was dried on the bottom of a glass flask. Mes-NaOH (pH 7.0) (20 mM, 9 ml) was added, and the suspension sonicated using a micro-tip for 3 × 20 s (power setting 6) with intervening cooling periods. The isolated Rieske protein and depleted cytochrome complex fractions were added to the lipid solution. The molar ratio of lipid to protein was 20, and the volume of buffer added to the lipid was calculated so that the final concentration of Triton X-100 (associated with the protein fractions) would be 0.01%, lower than the critical micellar concentration (CMC) of this detergent. The ratio of Rieske protein to depleted complex was 2:1 (mol Fe-S center: mol cytochrome f). The dilute mixture of proteins and lipids was then dialyzed for 20 h against the same buffer, containing Bio-Beads SM 2 (Bio-Rad). After dialysis the mixture was centrifuged at 60000 rpm in a Ti-60 rotor for 1 h. The supernatant was discarded and the pellet containing the proteoliposomes was resuspended in a solution of 30 mM octylglucoside, 0.5% Na cholate and 18% sucrose. This resuspended complex was used for activity measurements. All steps of this procedure were carried out at 4°C. As a control, we reconstituted a control cytochrome b_6-f complex into liposomes by the same procedure described above.

Oxidoreductase activity of the cytochrome complex, as well as the reactions of cytochrome b_6, were assayed according to Willms et al. [8], with duroquinol as electron donor and plastocyanin as acceptor. Cytochrome content was assayed by measuring reducing-minus-oxidized chemical difference spectra [8]. SDS-PAGE analysis was performed using the gel system of Laemmli [16], on a linear gradient slab gel from 10 to 15% polyacrylamide. EPR measurements of the Fe-S center concentration of the Rieske protein and the depleted complex were carried out as described by Hurt et al. [14]. Protein content was determined by the BCA method [17].

3. RESULTS

Isolation of the Rieske Fe-S protein from the spinach cytochrome b_6-f complex has previously been demonstrated [14]. However, we found that occasionally the isolated protein was degraded from a 20 kDa peptide to a 14 kDa species. Since this degradation occurred during the purification steps following hydroxyapatite chromatography, i.e. dialysis and DEAE-cellulose chromatography, we limited the original procedure to its first step only: chromatography on hydroxyapatite column in the presence of Triton X-100. The isolated protein migrated on SDS-PAGE as a single band with an apparent molecular mass of 20 kDa (fig.1, lane 3), and showed a typical Rieske Fe-S center EPR spectrum characterized by g values at 2.02 and 1.89 at liquid helium temperatures. The Rieske-depleted complex eluting from the column showed no traces of the Rieske protein by SDS-PAGE analysis (fig.1, lane 2), had no EPR signal of the Fe-S center and totally lost its oxidoreductase activity.

Attempts to reconstitute the Rieske protein back into the depleted complex by merely mixing the

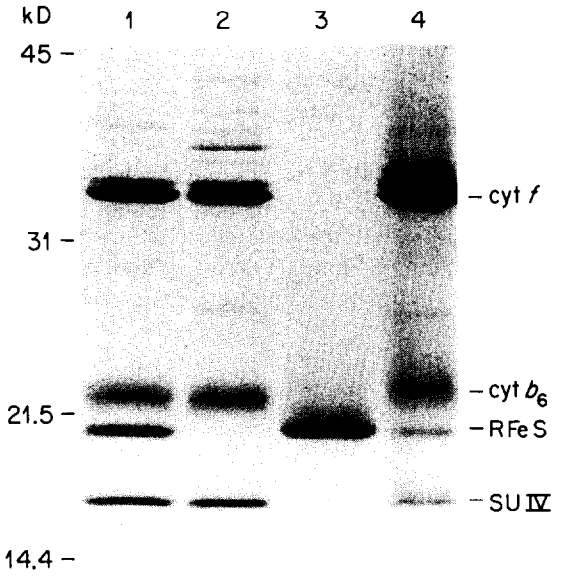

Fig.1. SDS-PAGE analysis of the Rieske-depleted and reconstituted cytochrome b_6-f complex. Samples were resolved on a 0.75 mm, 10–15% polyacrylamide slab gel. Lanes: 1, control cytochrome b_6-f complex; 2, Rieske-depleted complex; 3, Rieske Fe-S protein; 4, reconstituted Rieske-depleted complex + Rieske protein.

two protein fractions together were unsuccessful [14]. We therefore tested for an alternative reconstitution system. Phosphatidylcholine was first sonicated in buffer and the two protein fractions were then added to this solution. Following this dilution step, the concentration of Triton X-100 associated with the proteins dropped below the detergent CMC (<0.015%). The mixture was dialyzed for 20 h against buffer containing Bio-Beads SM 2, allowing the exchange of detergent with lipids and the absorbance of the Triton monomers outside the dialysis tube by the beads. The dialyzed mixture was then centrifuged at high speed and proteoliposomes were recovered in the pellet. These were resuspended in detergent solution and assayed for activity. As shown in fig.1, SDS-PAGE analysis of the preparation shows the Rieske protein has been reincorporated into the complex as a consequence of this procedure.

Depletion of the cytochrome b_6-f complex from the Rieske Fe-S protein totally abolished its plastocyanin-reduction activity and mixing the two protein fractions together could only stimulate its activity to a small extent (table 1). Reconstitution of the Rieske and the depleted fractions by the above procedure resulted in an active complex that could reduce plastocyanin at half of the rate of a control untreated complex. When the control complex was carried through the same reconstitution procedure, it lost about 50% of its oxidoreductase activity compared with the isolated complex, and showed a plastocyanin-reduction rate comparable to that of the reconstituted complex (table 1). The restored activity could not be accounted for by a lipid effect [13], since addition of lipids to the depleted complex itself did not stimulate the plastocyanin reductase activity. Changing the ratio of lipid to protein in the reconstitution system from 10:1 to 80:1 did not affect the activity of the reconstituted complex, while lowering the ratio of Rieske protein to depleted complex from 2:1 to 1:2 resulted in an inactive complex.

A partial reaction of the cytochrome complex was tested by monitoring the reduction of cytochrome b_6. Upon introduction of duroquinol, into a reaction mixture containing the control complex, a rapid reduction was observed, followed by a two-phase reoxidation (fig.2a). When the same complex was reconstituted into liposomes it showed different kinetics; the cytochrome was rapidly reduced, although to a lower extent, and apparently remained reduced (fig.2c). Depletion of the cytochrome b_6-f complex of the Rieske protein

Table 1

Reconstitution of electron transport in a Rieske-depleted cytochrome b_6-f complex

Conditions	Activity (μmol PC reduced/ nmol cytochrome f per h)
Control complex	30.0
Liposome-incorporated control complex[a]	16.3
Rieske-depleted complex	0.7
Rieske-depleted complex + isolated Rieske protein	3.0
Reconstituted Rieske-depleted complex + Rieske protein[a,b]	14.8

[a] Lipid/protein = 20:1 (mol:mol)
[b] Rieske-depleted/Rieske = 1:2

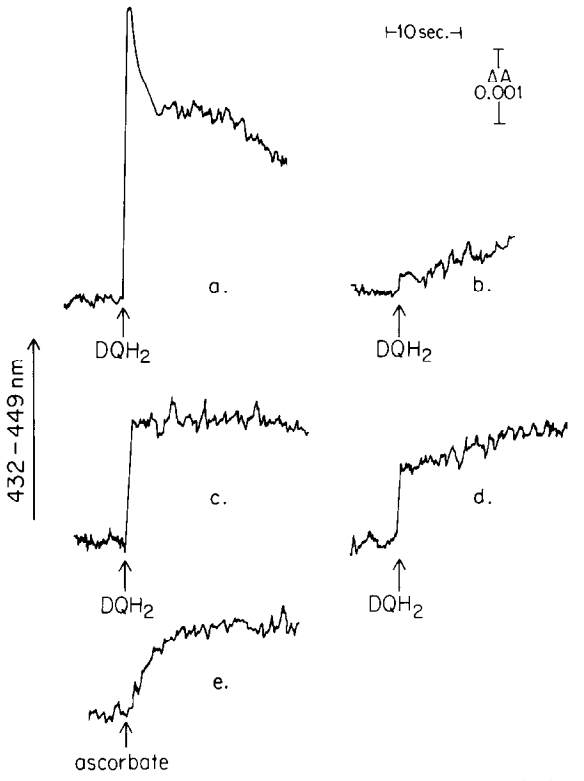

Fig.2. Redox reactions of cytochrome b_6 in a Rieske-depleted and reconstituted cytochrome b_6-f complex. Reduction of cytochrome b_6 was assayed as in [8]. (a) Control cytochrome b_6-f complex; (b) Rieske-depleted complex; (c) liposome-incorporated control complex; (d) reconstituted Rieske-depleted complex + Rieske protein; (e) ascorbate-induced absorbance change of a liposome-incorporated control complex.

totally abolished the fast reduction of cytochrome b_6 (fig.2b). Following reconstitution, the depleted complex showed a fast initial phase of reduction of cytochrome b_6, with no subsequent reoxidation (fig.2d), similar to the control (fig.2c) carried through the same procedure. At the wavelength pair used, there is some contribution from reduced cytochrome f and plastocyanin to the observed absorbance changes. We therefore estimated this contribution by monitoring the absorbance change upon addition of ascorbate, which reduces cytochrome f and PC but not cytochrome b_6. Using a control complex reconstituted into liposomes it was found that, although the total signal was comparable to that observed with duroquinol, the initial rapid reduction was appreciably lower (fig.2e).

The concentration of Rieske protein used for these reconstitution studies was estimated by means of EPR and actually represented the concentration of Fe-S centers. This was not necessarily the same as the protein concentration, since the protein concentration of the Rieske fraction was generally 3–4-fold higher than that based on EPR. This suggests that some of the Rieske molecules have lost their Fe-S centers during purification. Attempts to obtain a reconstitutively active Rieske fraction by other procedures were also made. Following the work of Shimomura et al. [18] on mitochondrial cytochrome b-c_1 complex, we resolved the cytochrome b_6-f complex into a Rieske Fe-S protein fraction and a Rieske-depleted complex by means of hydrophobic chromatography on a phenyl-Sepharose column. The isolated Rieske protein appeared to be pure but showed no Rieske Fe-S center EPR signal, and therefore could not be used for reconstitution studies.

4. DISCUSSION

Depletion of the cytochrome b_6-f complex from the Rieske protein by means of chromatography on a hydroxyapatite column in the presence of Triton X-100 resulted in the loss of oxidoreductase activity [14], as well as the ability to reduce cytochrome b_6 [15]. Previous attempts to reconstitute the resolved components into an active oxidoreductase proved unsuccessful [14]. Since both the Rieske protein and the depleted complex are associated with Triton X-100 micelles, and considering the relatively large mass of these Triton micelles [19], it is not surprising that the two protein fractions failed to reassociate with each other. Therefore, we set up an alternative procedure for reconstitution, which enabled us to carry out the removal of the Triton X-100 molecules, and their substitution by lipids. This method involved dilution of a mixture of the proteins and lipids in order to lower the Triton concentration below its CMC so it could be dialyzed out. To facilitate the dialysis, Bio-Beads SM 2, capable of binding Triton, were included in the dialysis buffer.

Upon reconstitution of the Rieske protein into a Rieske-depleted complex, the oxidoreductase activity of the complex was restored. The reactivated complex reduced plastocyanin at about the same rate as a control complex reconstituted into

liposomes by the same procedure, and the kinetics of cytochrome b_6 reduction were also similar. However, both activity and cytochrome b_6 reduction kinetics were somehow slower than those observed in the isolated complex. Two possibilities might account for these differences. Estimation of the Rieske protein concentration by both protein assay and EPR spectroscopy revealed a 3–4-fold difference in favor of the protein assay. This observation suggests a loss of Fe-S centers in some of the Rieske protein molecules. Therefore, not all of the reconstituted Rieske protein molecules are active in electron transport and this could lead to lower rates of plastocyanin and a lower extent of cytochrome b_6 reduction. A second possibility is that the prolonged reconstitution procedure destabilized one of the *b* hemes, resulting in a lower activity of the reconstituted complex. This possibility is supported by a recent observation that treatments, including pelleting the complex by high-speed centrifugation, can be correlated with loss of activity and the appearance of a high-spin EPR signal of a *b* heme with a peak at $g = 6$ [20]. This might explain the reduced activity of both reconstituted control and resolved complexes, since both are pelleted following their reconstitution into liposomes. The activity values of the reconstituted complexes reported here (40–50% of the isolated complex) are also comparable to those reported for the inactivated complex described by Nitschke and Hauska [20].

The role played by the Rieske protein in the oxidoreductase activity of the cytochrome b_6-*f* complex has been suggested based on the redox potentials of the different components of the complex. The Rieske protein is an essential link in the electron transfer chain leading to the reduction of plastocyanin as well as in the reduction of cytochrome b_6. Confirmation of this role has thus far been obtained in studies using PQ analogs interacting with the Rieske protein [4,21,22], thus inhibiting all activities. Depletion of the complex from Rieske also inhibited both plastocyanin [14] and cytochrome b_6 reduction [15], supporting its suggested role. The results presented in this work provide additional support for the necessity of the Rieske protein, since it can be clearly documented that its reconstitution into a Rieske-depleted complex can restore oxidoreductase activity as well as cytochrome b_6 reduction.

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