

Some Properties of Plastidic Cytochrome b-563

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Plastidic Cytochrome b-563

Cytochrome b-563 from spinach chloroplasts was isolated in a homogeneous and enzymatically active form. It has a low, but variable lipid content and a molecular weight of about 18,000 daltons.

Cytochrome b-563 is a tightly bound ("integral") protein of chloroplast thylakoids present in algae and higher plants. Apparently, it functions as a redox carrier in cyclic electron flow¹. Isolation, which has been reported^{2,3}, encounters the same difficulties as with the plastidic cytochrome b-559. For both proteins the preparative procedures have been improved by us^{4,5}, yielding cytochrome b-563 in a homogeneous and enzymatically active form.

The preparation was carried out with ethanol-extracted chloroplast material from spinach (*Spinacia oleracea*, strain Atlanta) as described⁴ (see refs. 4 and 5 for general methods and materials). In this case, however, the extracted chloroplast material equivalent to 100 mg of chlorophyll was suspended in about 12 ml of 4 M urea, including 2% Triton X-100 and 50 mM Tris-HCl buffer, pH 8.0 [N-tris-(hydroxymethyl)-aminomethane], taking care to omit any thiol-group containing reagents which deliberately leads to destruction of cytochrome b-559, but not of b-563. The suspension was gently sonified as published⁴, the resultant supernatant chromatographed on a Biogel A-1.5 m column (Biorad, Munich) as specified⁵ and eluted in the oxidized form with 4 M urea and 2% Triton X-100 buffered with 50 mM Tris-HCl, pH 8.0. The purest fraction (containing about 5 μ M cytochrome) was determined by the absorption spectrum of its oxidized and reduced form (Fig. 1) and separate protein determination. Denatured cytochrome had the α -band peak shifted to 561–562 nm.

Homogeneity was controlled by analytical disc gel electrophoresis according to⁶ as demonstrated in Fig. 2. To obtain the reproducible R_F value of 0.68, the gel had to be of 7.5% acrylamide only and to be polymerized by the catalytic riboflavin/tetramethyl-ethylene diamine (TEMED) couple. For molecular weight determination the protein was treated beforehand as indicated in the legend of

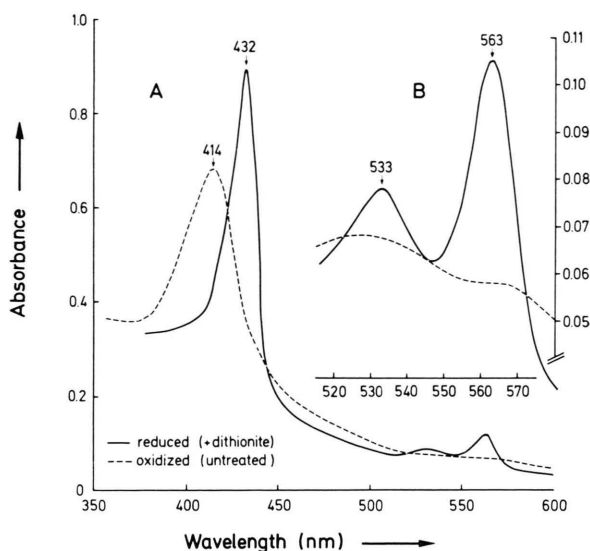


Fig. 1. Absorption spectra of isolated plastidic cytochrome b-563 from spinach in 4 M urea including 2% Triton X-100 and 50 mM Tris-HCl buffer, pH 8.0. An extinction coefficient of $20.7 \text{ mm}^{-1} \times \text{cm}^{-1}$, between 563 and 543 nm, was used⁴. The reduced form was produced with some particles of solid Na-dithionite added to a stoppered 1 ml cuvette.

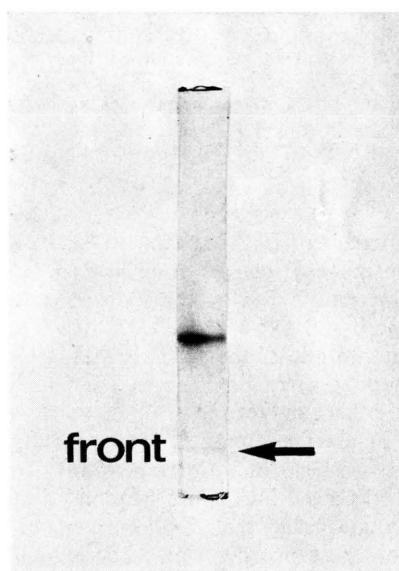


Fig. 2. Analytical polyacrylamide disc gel electrophoresis of spinach cytochrome b-563. The gel had 7.5% acrylamide and 0.5% Triton X-100. Electrophoresis was carried out for 5 h at 4 mA per gel tube in 10 mM Tris-HCl buffer, pH 8.0, including 80 mM glycine and 0.5% Triton X-100 with 30 μ g of protein applied per gel.

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Fig. 3, which caused more diffused bands in the gels as was observed with cytochrome b-559⁵. However, under the conditions noted also the



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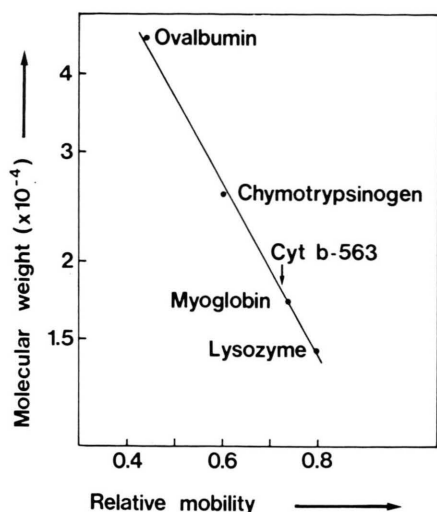


Fig. 3. Molecular weight determination of spinach cytochrome b-563 by disc gel electrophoresis. The protein was pretreated beforehand by 100 °C for 5 min in the presence of 1% sodium dodecylsulfate and 20 mM dithiothreitol, then incubated at 37 °C in the same medium for 12 h. The gel, 7.5% in polyacrylamide, contained 0.5% Triton X-100 and 4 mM dithiothreitol. Electrophoresis was done with 40 μ g of pretreated protein per gel at 8 mA per tube for 5 h in Tris-HCl buffer, pH 8.0, with 0.2% Triton X-100 and 0.1% sodium dodecylsulfate. The latter concentrations were chosen from trials with 0.1 to 0.5% of both components and found to be optimal for marker proteins and cytochrome b-563 as well. Runs were performed separately for cytochrome and the group of marker proteins, respectively. The molecular weights of the marker proteins are: ovalbumin, 45,000; chymotrypsinogen 25,600; myoglobin, 17,800; lysozyme, 14,000 (all purchased from Serva, Heidelberg).

marker proteins (although with difficulty for chymotrypsinogen) could be clearly distinguished allowing determination of the molecular weight of cytochrome b-563 to be $18,000 \pm 2,000$. This figure corresponds to one of the subunits having a 20,000 molecular weight, as determined previously in gel runs by other authors³. We could not detect separate bands in our gels and, therefore, not determine fractions of 9,600 and 6,000 molecular weight, as reported by them for spinach cytochrome b-563. In addition, the lipid content of several preparations was not constant (33%) as reported, but was found variable from 5–20%. This neither had an influence on the spectrum nor on enzymic activity.

The protein was readily reduced by ferredoxin, to which electrons were donated from NADPH

through ferredoxin-NADP reductase (EC 1.6.7.1) from the alga *Bumilleriopsis*. Details of this assay are described elsewhere⁷. The double reciprocal plot yielded straight lines between 0.13 and 1 μ M ferredoxin assayed with a K_m of 0.6 μ M for ferredoxin (Fig. 4). This is remarkably low and about the same value as found with soluble cytochrome c-553 from the alga just mentioned. This assay was not intended to indicate any physiological significance, but enzymatic reactivity of isolated cytochrome b-563 which was not shown by others³. This reactivity is lost by improper preparative performance, but in this case the protein can still be reduced by sodium dithionite.

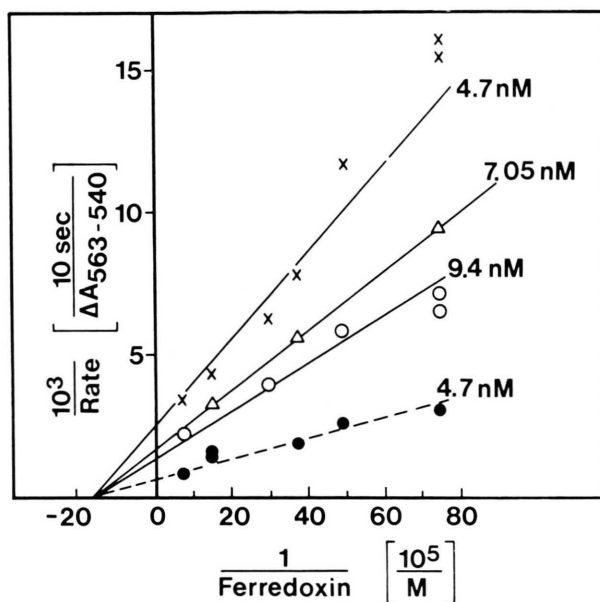


Fig. 4. Double-reciprocal plot of spinach cytochrome b-563 reduction by reduced ferredoxin. The reduction of ferredoxin was mediated by ferredoxin-NADP reductase and NADPH. The nmolar concentrations refer to the reductase; the cytochrome was 0.16 μ M (solid lines) and 0.4 μ M (dashed line). For the enzymatic assay the cytochrome b-563 was freed from urea by a 6×1 h dialysis vs 50 mM Tris-HCl, pH 8.0. Thereafter, the 2–3 μ M cytochrome solution, still containing approx. 1.5% Triton X-100, was frozen by liquid nitrogen. Storage at -80 °C preserved activity for 2–3 months.

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