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Purification, properties, and cellular localization of *Euglena* ferredoxin-NADP reductase

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Ferredoxin-NADP reductase from *Euglena gracilis* Klebs var. *Bacillaris* Cori purified to apparent homogeneity, yields a typical 36 kDa and an unusual 15 kDa polypeptide on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, exhibits a typical flavoprotein spectrum, contains FAD, and catalyzes NADPH-dependent iodonitrotetrazolium-violet diaphorase, NADPH-specific ferredoxin-dependent cytochrome-*c*-550 reductase and NADPH-NAD transhydrogenase activities. Rabbit antibody to the purified FNR blocks these activities specifically and also blocks the iodonitrotetrazolium-violet diaphorase activity of *Euglena* chloroplasts completely. The low iodonitrotetrazolium-violet diaphorase activity in the plastidless mutant, W_{10}BSmL, is mitochondrial and is not specifically blocked by the ferredoxin-NADP reductase antibody. Dark-grown non-dividing (resting) wild-type *Euglena* cells show a 4-fold increase in ferredoxin-NADP reductase activity during greening at 970 lx. Half of the low ferredoxin-NADP reductase activity in dark-grown cells is initially soluble, but by the end of chloroplast development nearly all of the enzyme is membrane-bound. The binding of ferredoxin-NADP reductase on exposure to light correlates with the extent of thylakoid membrane formation. Immunoblots of wild-type extracts during greening indicate that the 15 kDa polypeptide increases in the same manner as the extent of reductase binding to thylakoid membranes.

Introduction

Ferredoxin-NADP reductase is a flavoprotein that catalyzes the reduction of NADP using electrons from ferredoxin originating in photosystem I of photosynthetic electron transport [1,2]. The enzyme from higher plants, particularly spinach, has been well studied, and while some work has been done on the enzyme from various microorganisms [3–5] no reports of a highly purified enzyme from *Euglena* have appeared. The study of the *Euglena* enzyme is of some interest, since the technology is now available to prepare highly purified intact chloroplasts from this organism and studies of chloroplast development are readily carried out. In addition, *Euglena* ferredoxin-NADP reductase has been implicated in a blue-light system which results in the reduction of chloramphenicol [6]. Since a number of thylakoid constituents in *Euglena*, including ferredoxin-NADP reductase [7,8], have been found to be somewhat different from their counterparts in higher plants, showing different properties, low cross-reactivities with heterologous antibodies and often requiring greatly different methods for extraction and purification, we have undertaken to purify the *Euglena* fer-

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redoxin-NADP reductase to homogeneity in the present work. This was achieved without the use of detergents and, in the final steps, relied upon affinity chromatography on Reactive Blue-2 Sepharose, ATP-agarose and ferredoxin-Sepharose. The purified enzyme from *Euglena* was found to contain an unusual 15 kDa moiety in addition to the usual 36 kDa subunit. The presence of this 15 kDa subunit during development, the various activities and properties of the purified enzyme and the use of blocking antibodies to the purified molecule to localize the activity are subjects of the present paper. Brief reports of this work have appeared previously [9–11].

Materials and Methods

Wild-type and mutant cultures of *Euglena gracilis* Klebs var. *bacillaris* Cori were innoculated from slants of Hutner's pH 3.5 medium or from ongoing liquid cultures and were grown aseptically in 2-liter Erlenmeyer flasks containing 1 liter of Hutner's pH 3.5 medium at 26 °C [12]. Cultures illuminated with equal numbers of daylight and red fluorescent lamps were either placed on a rotary shaker at 110 cycles per min ('shaking cultures', 8600 lx) or kept without shaking with light from above and below ('standing cultures', 970 lx) [13]. Cultures kept in darkness were always shaken at 110 cycles per min. Unless otherwise specified, all manipulations involving dark-grown cells were performed under green safelights [14]. Light-induced chloroplast development in *Euglena* was studied in dark-grown non-dividing cells using previously described methods [15]; illumination was at 970 lx as above. The final cell density obtained was generally $(0.8–1.2) \cdot 10^6$ cells/ml.

Purification of ferredoxin-NADP reductase

Ferredoxin-NADP reductase was purified from wild-type cells of *Euglena gracilis* grown in standing culture in the light for 5–7 days after inoculation with 10–50 ml of a light-grown shaking culture in late logarithmic or early stationary phase. The cultures were filtered through four layers of cheesecloth to remove mucus and clumped cells prior to harvest; all subsequent manipulations were performed at 4 °C, unless otherwise indicated. The filtrate was centrifuged at $365 \times g$ for 5 min; the cell pellets were combined and washed by centrifugation with a solution containing 100 mM Tris-HCl (pH 8.0) and 1.0 mM EDTA. Ten liters of cell culture usually yielded 60–90 grams of packed cells. These were suspended to yield approx. 1 g of cell pellet per 2 ml of Tris-EDTA solution. At this point, in some experiments, the proteinase inhibitors ε-amino-*n*-caproic acid and PMSF were added at concentrations of 10 mM and 100 μM, respectively. When used, they were also included at these concentrations in all solutions throughout purification. The PMSF was first dissolved in a small volume of ethanol (100–300 μl) at room temperature, and was then added as a solution; the ε-amino-*n*-caproic acid was added as a solid, since it dissolves readily.

To the cell suspension on a magnetic stirrer was added chilled acetone (cooled below −20 °C in liquid N_2) to a final concentration of 80% (v/v). The acetone was added rapidly, so that the final temperature of the entire mixture did not exceed −15 °C. The mixture was allowed to stir for about 15 min (the temperature was about −5 to −8 °C). The mixture was then filtered by suction through a large Büchner funnel fitted with a piece of Whatman 3MM filter paper, and the green cell paste was allowed to dry under suction to a moist cake to help remove excess acetone. Filtration was usually performed in two or three batches, since after passage of 300–500 ml of the mixture, the rate of suction through the filter paper decreases significantly. The filter paper pads bearing the green acetone cakes were frozen in liquid N_2, the papers removed, and the frozen cakes were ground in a large mortar for a few minutes until the particle size was small and uniform; the material was then partially dried under a stream of cold air for 10–15 min. The greenish acetone powder was suspended in 150–250 ml of Tris-EDTA solution, the mixture was stirred overnight and was then centrifuged at $11\,960 \times g$ for 60 min. Solid ammonium sulfate was added to the supernatant fluid to yield 35% of saturation (70.5 g of solid ammonium sulfate added to 100 ml of liquid at 0 °C was taken as 100% saturation). The mixture was stirred for an additional 15 min and centrifuged at $11\,960 \times g$ for 15 min. Ammonium sulfate was added to the supernatant fluid to bring the

concentration to 70% of saturation; the pH was kept between 7.5 and 8.0 by the addition of either solid Tris base or dilute NaOH.

The mixture was allowed to stir for 30 min, and was then centrifuged at 11 960 × *g* for 20 min. The light-green pellet at the bottom of the tube or floating at the top is collected by aspirating off the surrounding solution. The pellets were suspended in a minimum (30–40 ml) of 10 mM Tris-HCl (pH 7.7) containing 0.3 M NaCl and dialyzed against 2 liters of this solution overnight. The contents of the dialysis bag were then centrifuged at 11 960 × *g* for 30 min, and the slightly turbid reddish supernatant fraction (about 30–50 ml) recovered. This was placed in an Amicon concentration chamber equipped with a PM 10 filter, and stirred under $2.7 \cdot 10^5$ Pa (40 lb/in^2) of pressure from an N_2 tank for 2–4 h until the volume of the solution was about 4–7 ml. This turbid, concentrated, dark-red solution was centrifuged at 11 960 × *g* for 15 min and the supernatant fluid was loaded onto a 100 × 2.5 cm Sephadex G-100 (120 mesh) column equilibrated in a solution containing 10 mM Tris-HCl (pH 7.7) and 0.3 M NaCl. The column was eluted with the same solution at a flow rate of about 40 ml/h. The reddish band containing cytochrome *c*-552 is eluted after 8–10 h, and signifies the end of elution (the gram molecular weight of cytochrome *c*-552 from *Euglena* is about 10 000 [16]).

Fractions were spot-tested for iodonitrotetrazolium-violet (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride) diaphorase activity, and the most active fractions centered on the activity peak (about 30–40 ml) were pooled and dialyzed against two changes of 2 liters each of 10 mM Tris-HCl (pH 8.0) for at least 4 h per change. The dialyzed material was applied to a Reactive Blue-2 agarose column 1.0 × 5.0 cm, previously equilibrated in 10 mM Tris-HCl (pH 8.0)), at a flow rate of 20 ml/h. The column was washed with 25 ml of 10 mM Tris-HCl (pH 8.0), and was then eluted with 20–30 ml of the same solution containing a 0–0.5 M NaCl gradient. The active fractions, which were eluted at about 0.3–0.4 M NaCl, appear yellowish, and were pooled and dialyzed against 1 liter of 10 mM Tris-HCl (pH 8.0) overnight. The dialyzed solution was applied to a 0.5 × 3.0 cm. ATP-hexane-agarose column, equilibrated in 10 mM Tris-HCl (pH 8.0), at a 10 ml/h flow rate. The column was washed with 10–20 ml of 10 mM Tris-HCl (pH 8.0), followed by 10 ml of a 0–0.2 M NaCl gradient in the same buffer. The yellow fractions (active in the iodonitrotetrazolium-violet diaphorase assay), eluted in about 80 mM NaCl, were pooled and dialyzed against 1 liter of 5.0 mM Tris-HCl (pH 8.0). The dialyzed material was applied to a 1.5 ml ferredoxin-Sepharose affinity column in a Pasteur pippette, at a flow rate of 10 ml/h. The ferredoxin-Sepharose column had been previously equilibrated in 5.0 mM Tris-HCl (pH 8.0). After sample application the column was washed in 5–10 ml of 5.0 mM Tris-HCl (pH 8.0), and 10–15 ml of a 0–0.2 M NaCl gradient in the same buffer was used for elution. The ferredoxin-NADP reductase activity is found in 4–5 tubes (containing 1 ml each), with the activity peak eluting at about 70–80 mM NaCl. These fractions, some of which contain the highly purified *Euglena* ferredoxin-NADP reductase, are stored at −20 °C and are used for further analysis. This procedure is referred to as the 'longer' purification method to distinguish it from our earlier purification method which did not incorporate the ATP-agarose and Reactive Blue-2 affinity steps. This 'shorter' method was otherwise identical to the longer one, and gave material of comparable purity, but in lower yield. Both purifications have been repeated several times; the longer purification is recommended for routine use.

Preparation of cells for various assays

This was done by centrifuging an aliquot of cheesecloth-filtered cell suspension in a Clay-Adams tabletop centrifuge at setting 5 for 5 min, the supernatant fluid was removed by aspiration, and the cell pellet was resuspended in 50 mM Tris-HCl (pH 8.0); the centrifugation and aspiration steps were repeated. The final cell pellet is called the 'washed pellet'.

Chlorophyll determination

Total chlorophyll ($a + b$) was determined as described previously [13].

Enzyme assays

Iodonitrotetrazolium-violet diaphorase activity.

The iodonitrotetrazolium-vilet diaphorase activity of the *Euglena* ferredoxin-NADP reductase was measured according to Zanetti [17] except that the final concentration of Triton X-100 was 0.11% (w/v). During purification, column fractions were routinely spot-tested for diaphorase activity as described previously [18].

Determination of total ferredoxin-NADP reductase activity in cell extracts: membrane-bound and soluble enzyme. The washed cell pellet (see preceding) was resuspended in 50 mM Tris-HCl (pH 8.0) at a concentration of $5 \cdot 10^6$ cells/ml, and was then sonicated in an icewater-bath twice for 20 s, each time at the maximum microtip setting with a Branson Sonifier Model 200. Aliquots of the cell sonicate were taken for determination of iodonitrotetrazolium-violet diaphorase activity; the presence of Triton X-100 in the assay facilitates determinations of activity in crude cell sonicates and makes all assays, whether of soluble or membrane-bound enzyme activities, fully comparable.

The crude sonicate was centrifuged at $11\,950 \times g$ for 30 min at 4° C. The supernatant fluid was removed and the pellet was resuspended in 1.0 ml of 50 mM Tris-HCl (pH 8.0). The activity in the supernatant fluid was a measure of soluble ferredoxin-NADP reductase activity, and the activity in the pellet represented membrane-bound ferredoxin-NADP reductase activity. The percentage of ferredoxin-NADP reductase activity found in the membrane-bound form was calculated as the activity in the pellet divided by the sum of the activities of the pellet and supernatant fractions, all multiplied by 100. The recovery of the activity in the two fractions was excellent; $102 \pm 5.1\%$ (mean ± S.E. for 24 samples) of the activity in the crude sonicate was recovered in the two fractions after centrifugation.

Ferredoxin-dependent NADPH-specific cytochrome-c-550 reductase and transhydrogenase activity. The ferredoxin-dependent NADPH-specific cytochrome-*c*-550 reductase activity was measured according to the method of Zanetti and Curti [19], using spinach ferredoxin (Sigma). The NADPH-acetylpyridine-NAD transhydrogenase activity of *Euglena* ferredoxin-NADP reductase was measured according to Keister et al. [20].

Extraction of the enzyme prosthetic group. Aliquots (100–300 µl) of fractions obtained from the ferredoxin-Sepharose affinity column containing the highly purified ferredoxin-NADP reductase (72.5 µg/100 µl) were dialyzed against 1 liter of 63 mM NH_4HCO_3 (pH 7.7) at 4° C for 6 h. The dialyzed enzyme samples were lyophilized to a yellowish powder and were kept in the dark at −20° C until used. All further analysis of samples was performed under dim room light.

Approx. 50 µl of the ammonium bicarbonate buffer was added to the lyophilized residue which was heated in boiling water for 3 min, cooled on ice, and centrifuged in a Beckman Model B microfuge for 5 min at 4° C. Alternatively, the lyophilized sample was resuspended in approx. 50 µl of methanol; the suspension was incubated on ice for 30 min and 50 µl of the NH_4HCO_3 buffer was then added. The suspension was centrifuged for 5 min in a Beckman Model B microfuge at 4° C. Authentic FAD and FMN (both from Sigma) were dissolved in 200–400 µl of 63 mM NH_4CO_3 (pH 7.7); these and the supernatant fractions from the enzyme extractions were subjected to electrophoresis on Whatman 3MM paper in 63 mM NH_4HCO_3 (pH 7.7) in darkness at 300 V for 2–3 h in an LKB Multiphor apparatus at 8–10° C. Flavins were located under longwave (365 nm) ultraviolet light by their yellow-green fluorescence.

Absorption spectra of oxidized and reduced Euglena ferredoxin-NADP reductase. Absorption spectra of the highly purified enzyme at 4° C were obtained with a Perkin-Elmer Model 552 double beam spectrophotometer. The serum-capped cuvette containing 1.0 ml of the buffer plus enzyme in a N_2 atmosphere was incubated for 30 min on ice in dim light prior to recording the spectrum. Reductant-containing solutions were transferred from serum stoppered tubes under N_2 gas by means of a gas-tight Hamilton syringe. When added, NADPH was present in 15-fold molar excess over purified enzyme.

Antibody and antigen preparation

Preparation of the 15 kDa subunit of ferredoxin-NADP reductase. Electroelution of proteins from polyacrylamide gels were performed according to Cunningham and Schiff [8] at room temperature with the following modifications: a 4% (w/v) acrylamide plug identical in composition to the

resolving gel solution of Laemmli [21] was used instead of an agarose plug; the gel slices containing the Coomassie-stained 15 kDa band of *Euglena* ferredoxin-NADP reductase were equilibrated for 1 h in 375 mM Tris-HCl (pH 8.6) buffer containing 1% (w/v) SDS; the molecular weight cutoff of the dialysis tubing was 6000–8000 and electrophoresis was conducted at 200 V for 6–12 h.

The eluted samples in the dialysis bags containing Coomassie dye and the electroeluted 15 kDa protein were lyophylized, resuspended in a minimum amount of 10 mM Tris-HCl (pH 8.0) containing 0.1% SDS, dialyzed overnight at 4°C against the same buffer, and stored at −20°C for further use.

Immunization regime. Antibodies were elicited against the native *Euglena* ferredoxin-NADP reductase (containing the native 36 and 15 kDa subunits) prepared by the shorter method and to the SDS-denatured ferredoxin-NADP reductase 15 kDa subunit alone (electroeluted from SDS gels). Enzyme from the the Reactive Blue-2 agarose step in purification was used to prepare 15 kDa subunit by electroelution from gels. Two 2 kg New Zealand White female rabbits were bled 1 week prior to injection of *Euglena* proteins to obtain control or 'preimmune' serum.

All steps up to the injection of the rabbits were performed on ice. For each injection of native ferredoxin-NADP reductase, 0.5 mg of protein dissolved in 0.5 ml of 10 mM Tris-HCl (pH 8.0) was mixed with 1.0 ml of cold Freund's complete adjuvant and emulsified in a Sorvall Omni-mixer at setting 3 for 3 min; the emulsion (1.5 ml) was injected into the rabbit via the toe pad. Three injections were administered at weekly intervals. Beginning 1 week after the third injection, the rabbit was bled at weekly intervals as long as the antibody titre remained high. Immunization with the 15 kDa subunit of the *Euglena* ferredoxin-NADP reductase was done in a similar manner. For each injection, 100–200 μg of electroeluted 15 kDa protein in 0.5 ml of 10 mM Tris-HCl (pH 8.0) containing 0.1% SDS (w/v) received additional SDS to yield a final concentration of 1.0% This solution was mixed with 1.0 ml of Freund's adjuvant, emulsified and injected as before, once a week for 3 weeks. The rabbits were bled on the 4th and 5th weeks, and then three further injections were administered over the next 3 weeks. The rabbit was bled weekly thereafter.

Before using sera, they were routinely treated to purify and concentrate the immunoglobulin G (IgG) using previously described methods [22]; note that the concentration of NaCl in the dialysis buffer should read 0.9% (w/v) and not 9.0%.

Organelle isolation. Pure, intact, mature chloroplasts of *Euglena* were isolated from light-grown wild-type cells as previously described [23]. The pure chloroplasts isolated form the gradients were washed free of Percoll by dilution of the suspension with a cold solution containing 50 mM Tricine-KOH (pH 7.8), 0.33 M sorbitol, 2.0 mM Na_2EDTA and 1.0 mM $MgCl_2$, followed by centrifugation at $1086 \times g$ for 5 min at 4°C. The chloroplast pellet was resuspended in the preceding solution and frozen at −20°C for further use.

Pure, intact mitochondria were prepared from the plastidless $W_{10}BSmL$ mutant of *Euglena* as before [24]. The 'band A' mitochondria isolated from the gradient were washed by dilution in a solution containing 25 mM Hepes-KOH (pH 7.4), 0.25 M sucrose and 5.0 mM Na_2EDTA and centrifugation at $11\,950 \times g$ for 10 min at 4°C. The mitochondrial pellet was resuspended in a small amount of the same buffer and frozen at −20°C for further use.

Electrophoresis. Stock solutions containing both acrylamide (Sigma) and *N,N*-methylene bisacrylamide (Sigma) were prepared at room temperature and stored according to the method of Chua [25]. Fully denaturing SDS-polyacrylamide gel electrophoresis was performed according to Laemmli [21] with modifications. Slab gels containing 12 or 15% (w/v) acrylamide in the resolving gel, either 13 cm wide × 10 cm long × 1.5 mm thick or 13 cm × 20 cm × 1.5 mm, were cast at room temperature and were allowed to remain at room temperature for at least 3 additional h. The stacking gels containing 4% (w/v) acrylamide were also cast at room temperature and allowed to remain at room temperature for 1 additional h. Gels were subjected to electrophoresis prior to application of samples (i.e., they were 'pre-run') for 1 h at 100 V. Electrophoresis of sample through the stacking gel was performed at 100 V for about 30 min and electrophoresis through the resolving gel was at 200 V for 90–120 min at room tempera-

ture. Samples were prepared for SDS-polyacrylamide gel electrophoresis after Laemmli [21] in a solubilization buffer containing 125 mM Tris-HCl (pH 6.8), 2.0% (w/v) SDS, 10% (w/v) glycerol, 0.01% (w/v) Bromophenol blue, and, when added, 2.0% (v/v) β-mercaptoethanol. Where indicated, samples were incubated for 1–2 min in a boiling water-bath. Samples for SDS-polyacrylamide gel electrophoresis were loaded at room temperature. Staining and destaining of gels was performed as before [8]. Gel scans to quantify relative amounts of Coomassie-stained proteins were done with a Guilford Model 2000 spectrophotometer equipped with a Model 2520 gel scanning apparatus [8].

Electroblotting and immunodetection of the 15 kDa subunit of the Euglena ferredoxin-NADP reductase. The methods used are based on those of Towbin [26] and Symington [27]. All subsequent steps were performed at 25 °C, unless otherwise indicated. After completion of SDS-polyacrylamide gel electrophoresis the resolving gel was soaked for 10 min in transfer buffer which contained 25 mM Tris-HCl (pH 8.3), 1.92 mM glycine and 20% (v/v) methanol. The gel was placed between filter paper and sponge pads containing a nitrocellulose sheet as before [26] and the sandwich was loaded in a 4-liter chamber filled with transfer buffer. Voltage was immediately applied from an EC Model 479 power source supplying 25 V. The starting current was about 250 mA and increased to about 500 mA during the 4 h required for transfer.

After transfer was complete, the nitrocellulose sheet, containing the proteins originally in the acrylamide gel, was incubated in blocking buffer containing 10 mM Tris-HCl (pH 7.4), 3% (w/v) bovine serum albumin, 0.25% (w/v) gelatin and 150 mM NaCl for 1 h at 40 °C. The nitrocellulose sheet was rinsed with 200 ml of glass distilled water, and was then incubated with 20 ml of a solution containing 50 mM Tris-HCl (pH 7.4), 5.0 mM EDTA, 0.05% (w/v) Nonidet P-40, 0.25% (w/v) gelatin, 150 mM NaCl, about 6 mg of rabbit anti-15 kDa ferredoxin-NADP reductase subunit IgG, and further supplemented with about 6 mg of goat IgG purified from goat serum by the method used for rabbit serum. After incubation with the nitrocellulose paper for 2 h at room temperature with shaking (40 cycles/min), the solution was poured off, and the paper was rinsed three times with glass-distilled water. The nitrocellulose was then incubated for 2 h in a solution of the same composition as the one used for the previous 2-h incubation, but without rabbit IgG, and containing horseradish peroxidase-conjugated goat-antirabbit serum (Boehringer-Mannheim) at 1 : 3000 (v/v) dilution. The nitrocellulose sheet was then rinsed with glass distilled water, and was incubated in a mixture containing 50 mM Tris-HCl (pH 7.4), 1.0 M NaCl, 0.4% (w/v) *N*-lauroylsarcosine (Sarkosyl), 5.0 mM EDTA, and 0.25% (w/v) gelatin for 2 h after which the nitrocellulose was rinsed three times in glass-distilled water and air dried.

The nitrocellulose sheet was then incubated in 100 ml of a solution containing 40 mM Tris-HCl (pH 7.4), 20% (v/v) methanol and 0.6 mg/ml 4-chloro-1-naphthol with shaking as before. Hydrogen peroxide at a final concentration of 0.015% (v/v) was added to initiate color development which was allowed to proceed for 10–30 min with shaking at room temperature until bluish banding appeared.

Preparation of the ferredoxin-Sepharose affinity gel. The ferredoxin-Sepharose affinity gel was prepared by the method of Shin and Oshino [28], with modifications based on information contained in the technical bulletin 'Affinity Chromatography' [29]. About 2.5 ml of spinach ferredoxin (Sigma) (containing 2.0 mg/ml) was dialyzed twice against 1 liter of a solution containing 0.1 M $NaHCO_3$ and 0.15 M NaCl at 4 °C. Approx. 0.4 ml of cyanogen bromide-activated sepharose 4B (Sigma) was swollen in 20 ml of 1.0 mM HCl for 15 min at room temperature. The swollen gel was washed on a fritted glass funnel with 200 ml of 1.0 mM HCl in several aliquots of 30–40 ml, using gentle suction. The acid-washed gel was suspended in 3–5 ml of 0.1 M $NaHCO_3$ (pH 8.3) containing 0.5 M NaCl (coupling buffer), gently suction filtered, resuspended in 1–2 ml of coupling buffer and added to the dialyzed solution containing 5 mg of ferredoxin in 1–2 ml. After incubation for 2 h at room temperature with moderate shaking (30–60 cycles per min), the gel was centrifuged from the reaction solution in a Clay-Adams table-top centrifuge at setting 1 for about

5 min. The pink loose pellet of ferredoxin-Sepharose gel was added to about 5 ml of a solution containing 0.2 M glycine, 0.1 M $NaHCO_3$ (pH 8.3) and 0.15 NaCl, and was shaken at room temperature for about 90 min. The material was then washed with about 50 ml of cold 10 mM Tris-HCl (pH 8.0) by suction filtration and could be used immediately for chromatography. The ferredoxin-Sepharose resin is not very stable at 4°C, and decayed within 1–2 days. Storage at −20°C in a solution containing 10 mM Tris-HCl (pH 8.0) and 50% (v/v) glycerol prevented the decay. The gel could be used once initially, and two more times after storage at −20°C. Before re-use, the column was regenerated by washing with several column volumes of 10 mM Tris-HCl (pH 8.0) buffer containing 0.5 M NaCl, and then equilibrated by passage of 10 column volumes of the same buffer without NaCl.

Cyanogen bromide cleavage of Euglena proteins. Cyanogen bromide cleavage of *Euglena* proteins was performed after the method of Piatigorsky [30] scaled down to accommodate microgram quantities of protein. The final powders, containing the CNBr-digested proteins, were resuspended in 25 μl of Laemmli solubilization buffer [21], vortexed thoroughly, immersed in a boiling water bath for 1–2 min, cooled and subjected to SDS-polyacrylamide gel electrophoresis at 25°C [21]; the gel dimensions were 60 × 80 × 1 mm. The stacking gel was 4% acrylamide, and the resolving gel was 15% acrylamide. Gels were stained with Coomassie blue and destained as above (see preceding). A control experiment showed that protein samples left in formic acid under identical conditions but without the addition of CNBr underwent no cleavage.

Other methods

Cell density was measured and thylakoids were prepared as before [8]. Samples were assayed for protein content by spectrophotometry at 750 nm according to Lowry et al. [31], as described by Schleif and Wensink [32] or, where indicated, by the method of Bradford [33]; bovine serum albumin (fraction V) was used as a calibration standard.

TABLE I

SUMMARY OF THE PURIFICATION OF FERREDOXIN-NADP REDUCTASE (FNR) FROM WILD-TYPE LIGHT-GROWN *EUGLENA* CELLS

Purification from acetone treatment of crude extract to homogeneity using the longer method. INT, iodonitrotetrazolium-violet.

Step	FNR activity (μmol INT reduced per min)	Protein (mg)	FNR spec. Act. (μmol INT reduced per min per mg protein)	Purification (-fold)	Recovery (%)	
Acetone fractionation	432	1304	0.33	0.0	100	
$(NH_4)_2SO_4$ fractionation	245	352	0.70	2.1	57	
Sephadex G-100 chromatography	219	105	2.1	6.4	51	
Reactive Blue-2 chromatography	125	11	11.3	34.8	29	
ATP-Agarose chromatography	79	4.8	16.5	50.7	19	
Ferredoxin-Sepharose chromatography [a]						
1. fraction 4	25	0.8	30.0	90.0	5.7	
fraction 5	11	0.2	49.7	150	2.4	16
2. fraction 4	28	0.7	37.4	113	6.4	
fraction 5	7	0.1	54.7	165	1.6	

[a] The material from the ATP-agarose step was divided in half, and each half was chromatographed separately (1 and 2).

Results and Discussion

The ferredoxin-NADP reductase from light-grown *Euglena* cells has been purified to homogeneity in the present work. A purification of 3–4-fold was routinely achieved by acetone fractionation of the crude extract. This, combined with the purification obtained in subsequent steps (Table I) results in an overall purification of 500–700-fold. The ATP-agarose affinity-chromatography step is probably effective in binding the reductase because of the similarity of this column to the adenylate portion of NADP, a substrate of the enzyme [34]; the ferredoxin affinity column also provides the specificity of substrate binding. The ATP-agarose and Reactive Blue-2 agarose steps are not essential and a more rapid purification-yielding enzyme of the same purity can be achieved by omitting them. However, the inclusion of these two steps results in higher final yields of the enzyme and extends the life of the ferredoxin affinity column. Both the scheme shown in Table I and the more rapid modification result in fully comparable enzyme preparations and these are used interchangeably in the present work. Similar enzyme preparations (not shown) can be obtained from light-grown cells of *Euglena* mutant G_1BUL, a mutant lacking antenna components [8,13], as well as from dark-grown wild-type cells. The method appears to be widely applicable, can be used with fairly low amounts of cells, or where the enzyme activity is somewhat lower than in light-grown wild-type cells.

An elution diagram of the Sephadex G-100 column used in a typical enzyme purification is shown in Fig. 1. The conditions used (a previous acetone step and the inclusion of 0.3 M NaCl in the column buffer) are known to favor the presence of the monomeric form of the enzyme from higher plants [35,36]. Under these conditions, most of the *Euglena* enzyme activity is eluted as a 49.4 kDa entity in a single symmetrical peak. A small amount of activity is present in the void volume, but these fractions are green and turbid and probably contain a small amount of enzyme bound to membrane fragments carried through from the previous step.

The enzyme fractions were subjected to SDS-

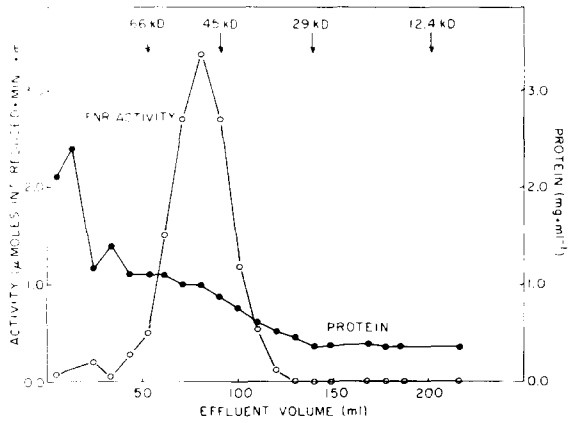

Fig. 1. Sephadex G-100 chromatography during purification of the *Euglena* ferredoxin-NADP reductase (FNR) from light-grown cells. The active fraction (35–75% of saturation from the ammonium sulfate step) was resuspended, dialyzed, concentrated and loaded onto a Sephadex G-100 column as described in Materials and Methods. The arrows at the top show the elution positions of the standards: bovine serum albumin, 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase 29 kDa; and Cytochrome *c*, 12.4 kDa. During chromatography, FNR emerges at 49.4 kDa in relation to the standards.

polyacrylamide gel electrophoresis after denaturation of the samples with SDS in the presence of mercaptoethanol (Fig. 2). The pattern indicates increasing purity; by choosing and pooling the best fractions from the ferredoxin affinity column, a homogeneous preparation with only minor contaminants is obtained (Fig. 2, lane 5); these correspond to the fractions with highest specific activity shown in Table I. The highly purified enzyme shows two major components, a polypeptide of 36 kDa and one of 15 kDa. These are not covalently linked, since SDS treatment alone in the absence of mercaptoethanol yields the same pattern. The highly purified enzyme from dark-grown wild-type cells and light-grown mutant G_1 yield similar patterns on SDS-polyacrylamide gel electrophoresis (not shown). The same pattern is obtained if the proteinase inhibitors phenylmethylsulfonyl fluoride and ε-amino-*n*-caproic acid are included throughout purification. There is minimal contamination with the 39 kDa entity in the final preparation, although this component is strongly represented in the final eluate from the ATP-agarose column and is also present in a number of fractions from the ferredoxin affinity column. If the material from the ATP-agarose step or the

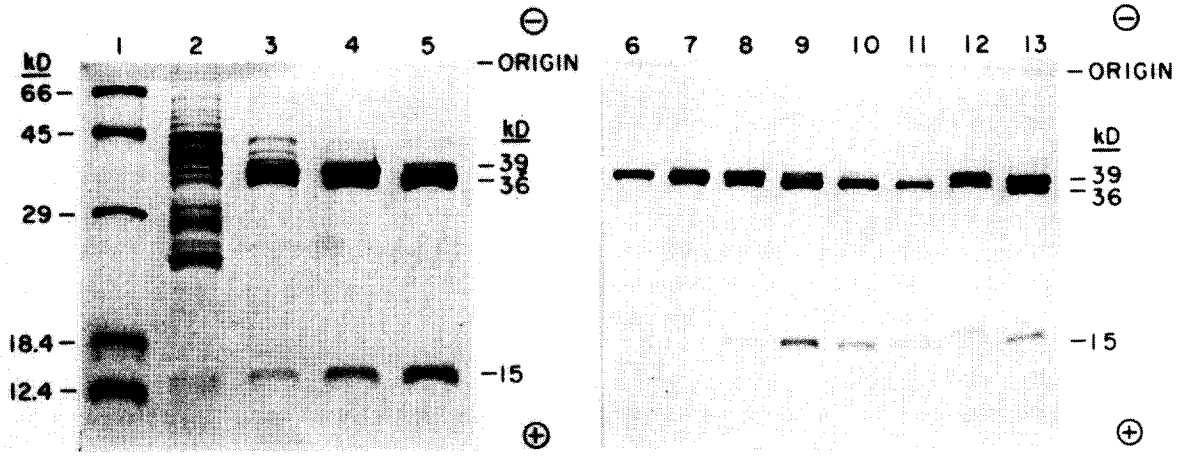

Fig. 2. Polypeptide profiles of *Euglena* ferredoxin-NADP reductase preparations. (Left) Polypeptide patterns obtained after SDS-polyacrylamide gel electrophoresis of *Euglena* FNR preparations at various stages of purification from light-grown wild type cells. Aliquots of 0.1 ml were taken at various steps during the purification of FNR, dialyzed against 5.0 mM Tris-HCl (pH 8.0) overnight, then lyophilized. Each residue was suspended in a solubilization buffer containing mercaptoethanol and heated, and a portion was applied to the gel. Lane 1: 2 μg each of standard molecular-weight markers including bovine serum albumin, 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 29 kDa; β-lactoglobulin, 18.4 kDa; and cytochrome *c*, 12.4 kDa. Lane 2: pooled peak from Sephadex G-100 (14.8 μg of protein). Lane 3: peak from Reactive Blue-2 agarose (10.6 μg of protein). Lane 4: peak from ATP-agarose (10.2 μg of protein). Lane 5: peak from ferredoxin-Sepharose (4.9 μg of protein). (Right) Polypeptide patterns obtained after SDS-polyacrylamide gel electrophoresis of fractions from the ferredoxin-Sepharose affinity column eluted in an expanded gradient of NaCl. FNR from the ATP-agarose step was applied to a ferredoxin-Sepharose column in 10 mM Tris-HCl (pH 8.0) and was eluted in a 0–0.2 M NaCl gradient in the same buffer. The peak fractions containing iodonitrotetrazolium-violet (INT) diaphorase activity were pooled, dialyzed against 10 mM Tris-HCl (pH 8.0) and were then reapplied to the column and eluted in an expanded (0–0.1 M NaCl) gradient in the same buffer; 15 fractions were collected of 1.0 ml each, and each fraction in the region of INT diaphorase activity (fractions 6–11) was dialyzed against 1.0 liter of 5.0 mM Tris-HCl (pH 8.0), lyophilized and resuspended in 100 μl of the same buffer. Aliquots of the resuspended fractions were used for SDS-polyacrylamide gel electrophoresis. The fraction (and μg of protein) applied to the gel in each case was as follows: lane 6, fraction 6 (4.5 μg); lane 7, fraction 7 (5.6 μg); lane 8, fraction 8 (6.4 μg); lane 9, fraction 9 (7.6 μg); lane 10, fraction 10 (4.8 μg); and lane 11, fraction 11 (3.0 μg). Lane 12 received the pooled INT diaphorase peak fractions from the first pass through the ferredoxin-Sepharose affinity column (7.0 μg of protein). Lane 13 received the pooled active fractions from the ATP-agarose column (6.3 μg of protein).

pooled fractions from the usual ferredoxin affinity column are subjected to fractionation on a ferredoxin affinity column with an extended salt gradient, the gel patterns shown in Fig. 2 are obtained. Lane 13 contains the material obtained from the ATP-agarose column in this experiment. Bands of 39, 36 and 15 kDa are present. After the first ferredoxin affinity column separation and a pooling of the fractions obtained, a similar pattern is seen (lane 12). When this material is refractionated on the second ferredoxin affinity column, fractions are obtained (lanes 6–11 in order of elution) which show a selective elution of the 39 kDa component first, followed by fractions containing decreasing amounts of the 39 kDa material and increasing amounts of the 36 and 15 kDa components. It is clear that the 39 kDa separates from the 36 and 15 kDa components which move together and are obtained as the final purified enzyme preparation. Fractions 6 and 7 are colorless and lack iodonitrotetrazolium-violet diaphorase activity. Fractions 8–11 show increasing amounts of iodonitrotetrazolium-violet diaphorase activity, increasing specific activity, and are characterized by a distinct yellow color expected for a flavoprotein. This evidence clearly shows that the *Euglena* ferredoxin-NADP reductase is composed of the 36 and 15 kDa entities and is distinct from the 39 kDa material. In a few preliminary experiments, cleavage of the highly purified enzyme with cyanogen bromide gives a different pattern of products on SDS-polyacrylamide gel electrophore-

sis from that given by fractions containing only the 39 kDa material (not shown) indicating that the two entities are not related in terms of common cleavage polypeptides.

The 15 kDa band on the gels prepared from highly purified ferredoxin-NADP reductase stains less intensely with Coomassie Blue than the 36 kDa band even after allowance is made for the difference in molecular weight. However, Sephadex Gel filtration at an earlier step of purification (Fig. 1) indicates a molecular weight of 49 400 which is in very good agreement with the value of 51 000 expected if the enzyme at this stage contained one each of the 15 and 36 kDa polypeptides. Either the enzyme loses some 15 kDa material during subsequent purification and/or the 15 kDa subunit stains anomalously on polyacrylamide gels. Anomalously low staining has been reported for hydrophobic polypeptides [37,38]. The ferredoxin-NADP reductase from higher plants, purified in a similar manner to that used in the present work (without the use of detergents) contains one or two polypeptides between 33 and 38 kDa. Since any one of these polypeptides is enzymatically active, the 36 kDa type of subunit contains the catalytic site. The ferredoxin-NADP reductase from *Euglena* contains only one type of 36 kDa subunit on gels subjected to SDS-polyacrylamide gel electrophoresis, and by analogy to higher plants, we infer that this is the catalytic subunit of the *Euglena* enzyme. The function of the 15 kDa moiety of the enzyme is not clear. Subsequent to our original report of the 15 kDa polypeptide in *Euglena* ferredoxin-NADP reductase [9], two other polypeptides which may be of a similar nature or function have been reported from higher plants. 'Connectein', 10 kDa by gel filtration, can be isolated from aggregates of spinach ferredoxin-NADP reductase containing 33 kDa subunits using isopropanol and may serve to bind these subunits together [39,40]. A 17.5 kDa polypeptide can be obtained from the spinach enzyme prepared by treatment of thylakoids with detergents [41,42]. The 17.5 kDa component can be separated from the 35 kDa subunit by treatment with EDTA [41] suggesting that they are bound by cationic linkages. It has been suggested that the 17.5 kDa polypeptide serves to bind the active subunit to the thylakoids; it differs, however, in amino-acid sequence from the 17 kDa polypeptide of the cytochrome b_6/f complex from spinach [42]. The 15 and 36 kDa components of *Euglena* ferredoxin-NADP reductase are tightly bound and, in our hands, are not separable by any of the aforementioned techniques; so far, only denaturation with SDS allows the two subunits to be separated. Thus, the binding of the smaller polypeptide in *Euglena* ferredoxin-NADP reductase appears to be different from that in spinach ferredoxin-NADP reductase. The tight binding of the 36 and 15 kDa entities in the *Euglena* enzyme probably allows the two to stay together throughout purification using acetone and standard purification techniques; similar techniques applied to spinach yields reductase without the smaller polypeptide.

As already mentioned, the highly purified active ferredoxin-NADP reductase preparations from *Euglena* are yellow in color. Fig. 3 shows the absorption spectrum of the highly purified *Euglena* enzyme from light-grown cells. This is a typical flavoprotein spectrum similar to that obtained for the enzyme from higher plants. The ratio of absorbance at 273 to that at 456 is 8.9; a value of 8.0 is reported for the enzyme from spinach [18] and a value of 9.1–9.4 for the enzyme from *Bumilleriopsis [5]*. Removal of the flavin portion of the

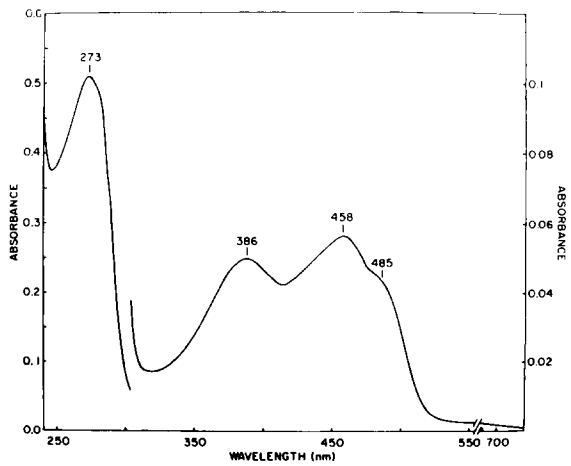

Fig. 3. Absorption spectrum of the highly purified *Euglena* ferredoxin-NADP reductase (FNR) isolated from wild-type light-grown cells by the longer purification method. The FNR was dissolved in 10 mM Tris-HCl (pH 8.0) and the mixture contained about 60 mM NaCl. The FNR concentration was 272 μg/ml.

Euglena enzyme yields only FAD and this, taken together with the spectrophotometric evidence, indicates that the *Euglena* enzyme, like the one from higher plants, contains one FAD per molecule of enzyme (assuming that the aromatic amino-acid contents of *Euglena* and higher plant enzymes are similar). The lack of FMN in the *Euglena* preparation rules out the presence of flavodoxin [44] a protein with a molecular weight similar to the 15 kDa moiety of the *Euglena* enzyme. Like ferredoxin-NADP reductase from other sources [45], the *Euglena* enzyme can be partially reduced with NADPH anaerobically with a lowering of the absorbance peak at 456 nm and the appearance of absorbance between 550 and 700 nm attributable to the semiquinone form of enzyme-bound FAD (data not shown). Treatment with sodium dithionite anaerobically brings about a more complete reduction of the enzyme. After reduction by NADPH or dithionite anaerobically, followed by exposure to air, the absorption spectrum indicates only a partial (about 50%) reoxidation of the enzyme preparation. This contrasts with the spinach enzyme where reoxidation is complete. This difference may be attributable to the presence of the tightly bound 15 kDa moiety in the *Euglena* enzyme and the absence of a small subunit from the spinach preparations used in this type of work; perhaps the 15 kDa moiety of the *Euglena* enzyme shields the reduced flavin from attack by oxygen or interferes with changes in the 36 kDa subunit that are associated with flavin oxidation.

The highly purified *Euglena* ferredoxin-NADP reductase catalyzes a number of reactions usually associated with ferredoxin-NADP reductase preparations (Table II). The *Euglena* enzyme catalyzes the reduction of iodonitrotetrazolium violet by NADPH (iodonitrotetrazolium-violet diaphorase activity), the ferredoxin-dependent reduction of cytochrome *c* by NADPH (NADPH-cytochrome-*c* reductase activity) and the reduction of acetylpyridine NAD by NADPH (NADPH-NAD transhydrogenase activity). All of these activities are blocked completely and selectively by antibody to highly purified native *Euglena* ferredoxin-NADP reductase (Table II).

As shown in Fig. 4, this antibody also inhibits the NADP-iodonitrotetrazolium-violet diaphorase activity of *Euglena* chloroplast lysates completely and selectively. Transhydrogenase activity in the same extracts is also completely blocked by the same concentrations of antibody (data not shown).

Euglena mitochondria also contain a small amount of NADPH-iodonitrotetrazolium-violet diaphorase activity, but this activity is not very sensitive to the antibody to the highly purified enzyme and the low amount of inhibition observed is non-specific, since pre-immune serum gives the same amount of inhibition (Fig. 4). The

TABLE II

SENSITIVITY OF VARIOUS ACTIVITIES OF THE PURIFIED *EUGLENA* FERREDOXIN-NADP REDUCTASE (FNR) TO ANTI-FNR SERUM

A frozen solution containing 43 μg of purified *Euglena* FNR (prepared by the shorter purification method from light-grown wild-type cells) in 100 μl of 10 mM Tris-HCl (pH 8.0) was thawed and kept on ice. 25-μl aliquots of this enzyme solution were mixed with 10–20 μl of preimmune or immune serum in 10 mM NaH_2PO_4 (pH 7.2) containing 0.15 M NaCl, or with phosphate/NaCl solution alone. Native FNR purified by the shorter method from light-grown wild-type cells had been used to prepare the antiserum. The volume of each sample was brought to 45 μl with glass-distilled water, and after incubation for 1 h on ice, aliquots were taken for the various assays. Activities of samples without serum protein were taken as 100% (controls). Control rates for iodonitrotetrazolium-violet (INT) diaphorase, NADPH-NAD transhydrogenase, and NADPH cytochrome-*c*-550 reductase activities were 19.1, 32.2 and 7.6 μmol substrate reduced/mg protein per min, respectively.

Serum	Serum protein (μg/45 μl)	Activity		
		INT diaphorase (% inhibition)	transhydrogenase (% inhibition)	cytochrome-*c*-550 reductase (% inhibition)
Preimmune	414	16	0	8
Immune	408	99	100	100

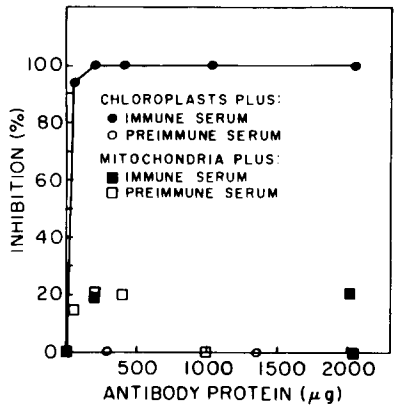

Fig. 4. Iodonitrotetrazolium-violet (INT) diaphorase activity in freeze-thawed *Euglena* chloroplasts and mitochondria in the presence of antiserum to the native purified *Euglena* ferredoxin-NADP reductase (FNR) from light-grown cells. A suspension of frozen purified *Euglena* chloroplasts was thawed and kept on ice. 20 μl of this suspension was diluted to 50 μl with a cold solution containing 10 mM Tris-HCl (pH 8.0). To this diluted suspension was added 50 μl of a solution containing 10 mM NaH_2PO_4 (pH 7.2), 0.15 M NaCl and either pre-immune serum, immune serum to the native purified FNR, or no serum whatever. The mixture was left on ice for 1 h. Aliquots (about 10 μl) were removed and assayed for INT diaphorase activity. A suspension of purified, frozen *Euglena* mitochondria was thawed and kept on ice. Samples were diluted and incubated as above for chloroplasts. Aliquots (10–20 μl) were removed and assayed for INT diaphorase activity. INT diaphorase activity measured in the absence of serum protein was taken as 100%. The rate in the absence of serum was 0.22 μmol INT reduced /min per mg protein for chloroplasts and 0.04 μmol INT reduced/min per mg protein for mitochondria.

data indicate that all of the NADPH-iodonitrotetrazolium-violet diaphorase activity of chloroplasts is due to the reaction catalyzed by ferredoxin-NADP reductase. The iodonitrotetrazolium-violet diaphorase activity of *Euglena* mitochondria is associated with some other enzyme activity (such diaphorases from mitochondria have been described previously in other systems [46]). By comparing ferredoxin-NADP reductase activities in chloroplasts and in cells on a chlorophyll basis, we conclude that 80–90% of the NADPH-iodonitrotetrazolium-violet diaphorase activity in *Euglena* cells is localized in the chloroplast and is, therefore catalyzed by ferredoxin-NADP reductase. Only 10–20% of the NADP-iodonitrotetrazolium-violet diaphorase activity is extra-chloroplastic and appears to be localized in the mitochondria; this activity is not attributable to ferredoxin-NADP reductase, leading to the conclusion that all of the ferredoxin-NADP reductase in the light-grown wild-type *Euglena* cell is localized in the chloroplasts, as in higher plants [47]. In agreement, the iodonitrotetrazolium-violet diaphorase activity of dark-grown resting cells of mutant W_{10}BSmL (which lacks plastids completely [48]) exposed to light for 96 h is only 10% of the activity found in dark-grown wild-type resting cells exposed to light for 96 h [11]. As would be expected, the mutant cells lacking plastids have the low level of iodonitrotetrazolium-violet diaphorase activity expected for mitochondria, while the wild-type cells with fully developed chloroplasts have, in addition, the very much greater activity attributable to the plastids.

In order to study the extent to which ferredoxin-NADP reductase is bound to membranes during chloroplast development, we again employed dark-grown resting wild-type cells (Fig. 5). As previously found [6,11,49] the 3–4-fold increase in

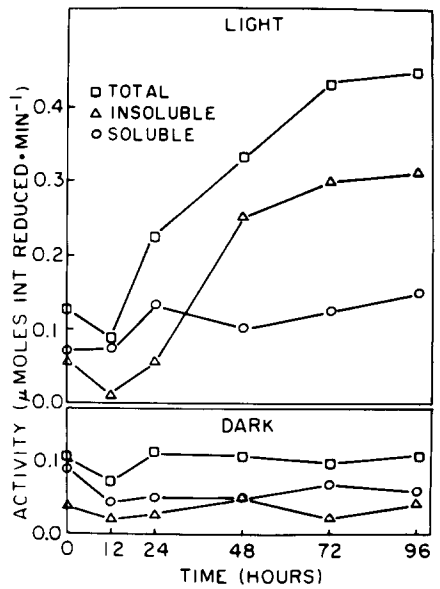

Fig. 5. The distribution of ferredoxin-NADP reductase (as iodonitrotetrazolium-violet diaphorase activity) between soluble and insoluble fractions from dark-grown resting wild-type *Euglena* cells exposed to light or kept in darkness. Samples of cell culture were harvested and washed at the appropriate times, then sonicated and assayed for total activity. The cell sonicate was centrifuged to separate soluble and insoluble fractions which were then assayed separately.

ferredoxin-NADP reductase activity during chloroplast development is strictly light-dependent. In the dark-grown cells, about 50% of the enzyme is found to be associated with membranes and 50% is freely soluble under our conditions. As light-induced chloroplast development proceeds, more and more activity is found in the membrane-bound fraction until, by the end of development, most of the activity is bound Since the soluble fraction increases only slightly during chloroplast development, it appears that most of the increase in activity that occurs during development results from an increase in the insoluble or membrane-bound fraction. The results with mutants (data not shown) support the correlation between chloroplast development and the increase in bound ferredoxin-NADP reductase. Dark-grown resting cells of the bleached mutant W_3BUL which contain only rudimentary proplastids [50] show ferredoxin-NADP reductase levels and degree of binding of the activity to membranes that are similar to those found in dark-grown resting wild-type cells. There is no appreciable increase in the usual plastid parameters when dark-grown resting cells of W_3 are illuminated and no increase in ferredoxin-NADP reductase activity or change in the amount bound is observed. Dark-grown resting cells of mutant G_1BU which contain fairly normal proplastids have ferredoxin-NADP reductase levels and binding of ferredoxin-NADP reductase

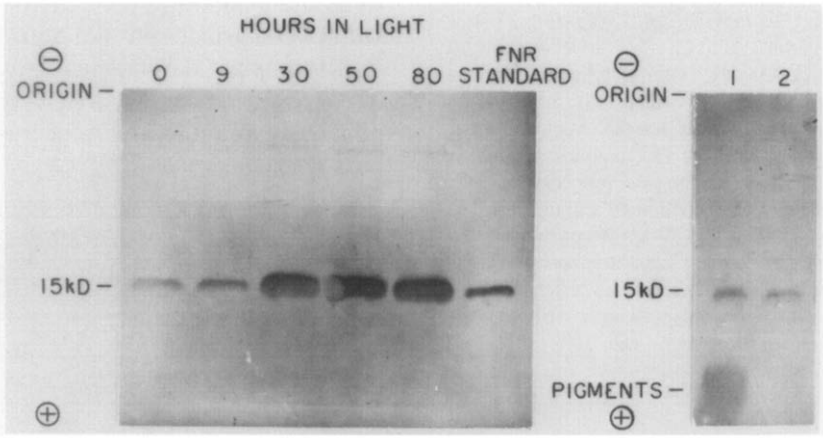

Fig. 6. Immunoblots of the 15 kDa polypeptide of the *Euglena* ferredoxin-NADP reductase (FNR) from resting dark-grown wild-type cells exposed to light to induce chloroplast development, and from thylakoids isolated from light-grown wild-type cells. (Left) The 15 kDa polypeptide of the *Euglena* FNR during light-induced chloroplast development in dark-grown wild-type resting cells. Dark-grown resting cells of *Euglena* were exposed to 90 ft.c of light. At the times indicated, samples of cell culture were removed and processed as for enzyme purification up to the acetone step except that the acetone precipitate was collected by centrifugation at $2987 \times g$ for 5 min at 2° C. The '0 hour' sample was processed through acetone extraction under green safelights and then brought into dim room light; the other samples were extracted and processed in dim room light. The acetone powders were resuspended in 100 mM Tris-HCl (pH 8.0) containing 1.0 mM EDTA, incubated overnight at 4° C, and centrifuged at $12350 \times g$ for 15 min. The supernatant fractions containing the FNR were dialyzed against 10 mM Tris-HCl (pH 8.0) overnight at 4° C and lyophylized. The samples were resuspended in a small volume of 10 mM Tris-HCl (pH 8.0), and aliquots containing equal amounts of protein were mixed 1:1 (v/v) with 2×Laemmli solubilization buffer (with β-mercaptoethanol) and were loaded in wells of a polyacrylamide gel. The samples were subjected to SDS-polyacrylamide gel electrophoresis and were electrophoretically transferred to nitrocellulose, where they were subjected to immunoblot analysis using antiserum elicited by the SDS-denatured purified 15 kDa polypeptide of the FNR. The amount of cell protein loaded per well for each sample was 70 μg. The FNR standard was purified FNR from the ATP-agarose step in the longer purification method (3.5 μg of protein). Protein determinations in buffer extracts were performed according to Bradford. (Right) The presence of the 15 kDa polypeptide in *Euglena* thylakoids prepared from light-grown wild-type cells. Thylakoids prepared from light-grown wild-type *Euglena* cells were solubilized and subjected to SDS-polyacrylamide gel electrophoresis, the separated polypeptides transferred to nitrocellulose, and immunoblotted using anti-15 kDa antiserum as above. Lane 1, thylakoids containing 6 μg chlorophyll (about 30 μg of thylakoid protein); lane 2, purified FNR from the ATP-agarose step in the longer purification method, (3.5 μg of protein). The material at the bottom of lane 1 is green and contains free pigments.

activity similar to those found in dark-grown resting wild-type cells. On illumination, cells of G_1 form less chlorophyll *a* than wild-type cells, have extremely low levels of chlorophyll *b* and lack antenna-related components such as the light-harvesting chlorophyll *a/b* protein complex and the chlorophyll protein Ia complex, although the chloroplasts formed show normal levels of photosynthesis in saturating light [8,13]. In this mutant, ferredoxin-NADP reductase activity increases about 2-fold on illumination and shows binding kinetics similar to wild-type cells during chloroplast development. Thus, the binding appears to be a property of the newly formed ferredoxin-NADP reductase activity and is probably related to the increasing availability of new membrane sites for binding as the total amount of thylakoid material increases during development [51]. As might be expected, the degree of binding appears to be independent of the formation of other thylakoid components such as the antenna complexes and chlorophyll *b* and does not require the formation of normal levels of chlorophyll *a*.

Buffer extracts of acetone powders made from dark-grown resting cells exposed to light were subjected to SDS-polyacrylamide gel electrophoresis, followed by electrophoretic transfer to nitrocellulose, treatment with antibody to the SDS-denatured 15 kDa component of the ferredoxin-NADP reductase and visualization with peroxidase (Fig. 6). The left-hand side of the figure shows that the 15 kDa moiety of the enzyme is detectable in dark-grown resting cells in agreement with our finding of a 15 kDa moiety in the enzyme purified from dark-grown cells (see above). Upon illumination of these cells, the 15 kDa moiety appears to increase in amount, at least to a first approximation. These data indicate that the 15 kDa component is a normal component of the enzyme that appears along with ferredoxin-NADP reductase activity and that it is not formed as an artifact of enzyme purification. The pattern of increase of the 15 kDa moiety is consistent with the pattern of increase in enzyme binding seen during chloroplast development (Fig. 5) and the possibility remains that the 15 kDa polypeptide may be a thylakoid binding protein for the 36 kDa catalytic moiety of the enzyme as has been suggested for the 17.5 kDa moiety of the spinach ferredoxin-NADP reductase prepared with detergents [41,42]. The right-hand side of Fig. 6 shows that the 15 kDa component is detectable in purified thylakoid preparations from light-grown cells using the same antibody methods and confirms that the 15 kDa moiety is a normal thylakoid component as would be expected from its association with ferredoxin-NADP reductase. In agreement, ferredoxin-NADP reductase containing both 36 and 15 kDa subunits can be isolated starting from *Euglena* thylakoid membranes instead of whole cells (not shown).

Euglena ferredoxin-NADP reductase is unusual, then, in having two subunits that are so tightly-associated that they remain together throughout purification by conventional techniques. Further studies of the significance of this association will be greatly facilitated if conditions can be found for the dissociation of the two moieties without denaturation.

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