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IDENTIFICATION AND CHARACTERIZATION OF ESSENTIAL MITOCHONDRIAL ACCESSORY PROTEIN COMPONENTS*

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In plants, the last step of the biotin biosynthetic pathway is localized in mitochondria. This chemically complex reaction is catalyzed by the biotin synthase protein, encoded by the *bio2* gene in *Arabidopsis thaliana*. Unidentified mitochondrial proteins in addition to the *bio2* gene product are obligatory for the reaction to occur. In order to identify these additional proteins, potato mitochondrial matrix was fractionated onto different successive chromatographic columns. Combination experiments using purified Bio2 protein and the resulting mitochondrial matrix subfractions together with a genomic based research allowed us to identify mitochondrial adrenodoxin, adrenodoxin reductase, and cysteine desulfurase (Nfs1) proteins as essential components for the plant biotin synthase reaction. *Arabidopsis* cDNAs encoding these proteins were cloned, and the corresponding proteins were expressed in *Escherichia coli* cells and purified. Purified recombinant adrenodoxin and adrenodoxin reductase proteins formed *in vitro* an efficient low potential electron transfer chain that interacted with the *bio2* gene product to reconstitute a functional plant biotin synthase complex. Bio2 from *Arabidopsis* is the first identified protein partner for this specific plant mitochondrial redox chain.

Biotin (vitamin B8) is a water-soluble molecule acting as a cofactor for a small number of enzymes involved in carboxylation, decarboxylation, and transcarboxylation reactions that are concerned with fatty acid and carbohydrate metabolism (1, 2). The biotin biosynthesis pathway described in bacteria is conserved in plants, and the last step of this pathway, undoubtedly the most complex, involves the biotin synthase protein, the *bio2* gene product in *Arabidopsis thaliana* (3–5). The fascinating reaction catalyzed by biotin synthase, *i.e.* the insertion of a sulfur atom between the unactivated methyl and methylene carbon atoms adjacent to the imidazolidone ring of dethiobiotin (DTB)[1] (Scheme 1), still remains an enigma for chemists and biologists. Recently, both the structure of the iron-sulfur centers of the enzyme and some aspects of the catalytic mechanism of the reaction (AdoMet dependence, sulfur atom donor, and electron donating system) have been unraveled by further studies essentially realized in *Escherichia coli*. First, biotin synthase (*bioB* gene product in *Escherichia coli*) is a dimeric enzyme of 76 kDa with a novel iron-sulfur center organization. Indeed, it is evolved to accommodate simultaneously two different oxygen-sensitive clusters, one $[4Fe\text{-}4S]^{2+}$ and one $[2Fe\text{-}2S]^{2+}$ per monomer, with unique roles in catalysis (6). The [4Fe-4S] could be ligated by three cysteines of a CX_3CX_2C box, and a fourth, still unidentified ligand (7–9). More recently, Ugulava *et al.* (10) proposed that the [2Fe-2S] center could be ligated at an alternative site not previously occupied by the [4Fe-4S] center. Second, the catalytic mechanism of biotin synthase reaction entitles the classification of this enzyme among the "radical AdoMet superfamily" (11, 12). Indeed, during the biotin synthase reaction, a radical species is generated by reductive cleavage of AdoMet (13). This radical species is necessary to permit the introduction of the sulfur atom into DTB. Cysteine is very likely the initial source of the sulfur atom for the reaction (14), but recent work provides evidence that the immediate sulfur donor is biotin synthase itself, at least *in vitro* (15, 16). Finally, a physiological reduction system is indispensable for the enzyme activity, whose role is to reduce AdoMet, through the [4Fe-4S] center, to generate the 5′-deoxyadenosyl radical. In *E. coli*, it consists of flavodoxin, flavodoxin (ferredoxin)-$NADP^+$ reductase, NADPH, and possibly another FMN-containing flavoprotein, MioC (14, 17–19). The reported activities with well defined assay mixtures rarely exceed 1 nmol of biotin/nmol of monomer. This failure to obtain multiple turnovers has sometimes been put forward to propose that biotin synthase is not an enzyme but a reactant. It has also been suggested that either BioB is irreversibly inactivated during the reaction by some undefined mechanism or that an important cofactor of the protein is missing in the *in vitro* assay mixtures. Importantly, Ollagnier-de-Choudens *et al.* (20) recently demonstrated that 5′-deoxyadenosine, a product of the reaction, is a very strong inhibitor of biotin formation *in vitro*, thus providing a convenient explanation for the lack of multiple turnovers.

In plants, we have recently described the first biochemical characterization of the biotin synthase reaction (21). The *bio2* gene product is a homodimer of 78 kDa presenting a high

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AY074925, AY074926, AY074927, and AF229854.

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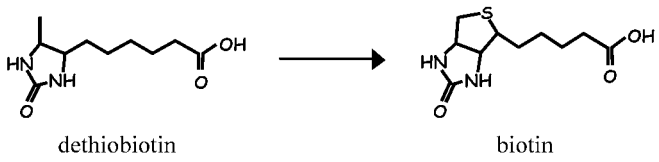
[1] The abbreviations used are: DTB, dethiobiotin; AdoMet, *S*-adenosylmethionine; Adx, adrenodoxin; AdxR, adrenodoxin reductase; Fdx, ferredoxin.



SCHEME 1

amino acid sequence similarity with the bacterial protein (4). Therefore, the CX_3CX_2C box is conserved and probably implied in an iron-sulfur ligation. Indeed, as its bacterial counterpart, the aerobically purified enzyme contains a $[2Fe\text{-}2S]^{2+}$ center according to the UV-visible spectrum (22). Moreover, the catalytic mechanism of the reaction is AdoMet-dependent, and the synthesis of biotin is inhibited by acidomycin, a structural analogue of biotin (21). Like its bacterial counterpart, the *bio2* gene product needs additional protein factors to function. Bacterial accessory proteins implied in the biotin synthase reaction efficiently complemented the plant enzyme (21). Our previous study has also shown the importance of mitochondria in the plant biotin synthesis. Therefore, we postulate that a mitochondrial physiological electron transfer system is required for plant biotin synthase reaction. It is probably similar to the system found in *E. coli* but implies different proteins because no flavodoxin has been documented in plants.

In the present work, we report the identification and the characterization of mitochondrial accessory proteins involved with *bio2* gene product in the plant biotin synthase reaction, including a low potential electron transfer chain. Cloning, expression, and purification of each of these proteins allowed the reconstitution, for the first time, of an *in vitro* functional plant biotin synthase complex.

EXPERIMENTAL PROCEDURES

Reagents—D-[8,9-^{3}H]Biotin (42 Ci/mmol) was purchased from Amersham Biosciences. (+)-[(6*R*),9-^{3}H]DTB (2 Ci/mmol) was custom-synthesized by PerkinElmer Life Sciences. Lactobacilli Man-Rogosa-Sharpe broth, Micro Inoculum broth, and dehydrated biotin assay medium were from Difco. Isopropylthio-β-D-galactoside was from Bioprobe Systems (Montreuil, France). His-binding resin was from Qiagen. Affi-Gel-10 and a molecular mass marker protein mixture for SDS-PAGE were purchased from Bio-Rad. All other biochemicals were obtained from Sigma and were the purest grade available.

Plant Material, Bacterial Strains, and Plasmids—Potato tubers (*Solanum tuberosum* L.) were obtained from a local market. The biotin auxotroph *Lactobacillus plantarum* (ATCC 8014) was obtained from the American Type Culture Collection (Manassas, VA). DNA manipulation was performed in *E. coli* DH5α cells (Invitrogen). Expression vectors pET28b(+), pET29a(+), pET21a(+), and the host strain *E. coli* BL21 (DE3) were from Novagen. Thermo-competent cells of this strain, prepared according to the method described in Ref. 23, were transformed with the different expression vectors used. The plasmid pBkat37 (carrying the molecular chaperones *GroES* and *GroEL* genes) (22) and the plasmid pSBET (24) (providing Arg triplets AGA and AGG frequently found in plant genes and in low abundance in *E. coli*) were used for optimal expression and correct folding of the recombinant proteins when indicated.

Preparation of Purified Mitochondria—Potato tuber mitochondria were purified using Percoll gradients (25). Intact mitochondria were lysed in buffer A (50 mM Tris-HCl (pH 8.0), 10% (w/v) glycerol, 1 mM dithiothreitol, 150 mM KCl, 5 mM 6-aminohexanoic acid, 1 mM benzamidine HCl, and 1 mM phenylmethylsulfonyl fluoride) by sonication with a Vibra-cell disrupter (Sonics and Materials, Danbury, CT). The suspension was then centrifuged (100,000 × *g*, 20 min). The pellet and the supernatant comprised the mitochondrial membranes and the soluble fraction (matrix), respectively. Mitochondrial matrix (4 g of proteins) was concentrated (20 mg protein/ml) using Jumbosep-3 tubes (Filtron).

Mitochondrial Matrix Fractionation—Mitochondrial matrix (400 mg of proteins) was loaded onto a gel filtration column (Superdex S200 Fast Flow, 2.6 cm × 60 cm, Amersham Biosciences) equilibrated with buffer A. Proteins were eluted with 320 ml of buffer A (flow rate 2 ml/min). Eighty fractions of 4 ml were collected and combined into four proteinaceous pools (pool I, fractions 1–40; pool II, fractions 41–50; pool III, fractions 51–58; pool IV, fractions 59–80). Each pool was concentrated using Jumbosep-3 tubes (Filtron) and desalted on a PD10 Sephadex G-25(M) column (Amersham Biosciences). The gel filtration column chromatography was repeated 10-fold in order to fractionate 4 g of mitochondrial matrix proteins.

Gel filtration pool IV (180 mg of proteins) was applied to a DEAE-Sepharose Fast-Flow column (2.6 × 12 cm, Amersham Biosciences) equilibrated with buffer B (50 mM Tris-HCl (pH 8.0), 10% (w/v) glycerol, 1 mM dithiothreitol). The column was washed with 50 ml of buffer B. Elution was then performed with a linear gradient of KCl from 0 to 0.3 M (300 ml) and 0.3 to 0.5 M (50 ml) in buffer B (flow rate 2 ml/min). One hundred fractions of 4 ml were collected. A 100-μl aliquot of each 4-ml fraction was assayed for biotin-forming activity in combination with pure Bio2 and gel filtration pool II (see "Results"). Fractions eluted with 80–150 and 350–450 mM KCl were required to restore biotin synthase activity. Separate pools of these fractions were concentrated using Jumbosep-3 tubes and desalted on a PD10 Sephadex G-25(M) column. The DEAE-Sepharose column 350–450 mM KCl eluted pool (10 mg protein) was further fractionated on a hydroxylapatite Bio-Gel HTP column (5 × 1 cm, Bio-Rad) equilibrated previously with buffer C (50 mM potassium phosphate (pH 6.8), 1 mM dithiothreitol). Proteins were eluted with a linear gradient of phosphate buffer from 0 to 100 mM potassium phosphate (100 ml) in buffer C (flow rate 0.5 ml/min). Fifty fractions of 2 ml were collected. A 50-μl aliquot of each 2-ml fraction was assayed for biotin-forming activity in combination with pure Bio2, gel filtration pool II, and DEAE-Sepharose column 80–150 mM KCl eluted pool (see "Results"). The pooled active HTP fractions (0.7 mg of protein) were concentrated using Macrosep-3 tubes, desalted, and stored at −80 °C.

Gel filtration pool II was fractionated onto a DEAE-Sepharose Fast-Flow column. The chromatographic conditions were the same as those described previously for fractionation of gel filtration pool IV. A 100-μl aliquot of each eluted fraction was assayed for biotin-forming activity in association with purified recombinant Bio2, Adx1, and AdxR proteins (see below for definitions). Active fractions were pooled, concentrated using Macrosep-10 tubes, and stored at −80 °C until use.

Engineering of Expression Vectors—The oligonucleotide primers utilized in this study (synthesized by MWG Biotech) are listed in Table I. For protein expression, a*dxR*, *adx1*, *adx2,* and *nfs1* cDNAs were cloned into pET expression vectors (Table I). Full-length cDNAs were obtained by PCR amplification of an *A. thaliana* (var. Columbia) cDNA library constructed in pYES (26). The PCR fragments were cloned into the cloning vector pPCR-Script (Stratagene) or pCR4Blunt-TOPO (Invitrogen) according to the manufacturer's instructions. All the cDNAs were sequenced, and the resulting sequences were submitted to the GenBank™ Data Bank. After proper restriction enzyme digestion, DNA fragments containing the coding sequences were subcloned into different pET expression vectors (Table I). The *E. coli* expression vector pET28b(+) was used to place a His_6 tag at the NH_2 terminus of the polypeptide.

Arabidopsis bio2 cDNA cloning has been described previously (22). The *Nde*I-*Bam*HI DNA fragment containing the *bio2* coding sequence was subcloned into the plasmid pET28b(+) in-frame with an NH_2-terminal His_6 tag coding sequence, yielding the plasmid pET28-*bio2*.

Expression of Recombinant Proteins—For correct Bio2 and Adx1 folding, BL21 strains containing the plasmid pET28-bio2 or pET28-adx1m were transformed with the pBkat37 vector, whereas efficient expression of Nfs1 protein was achieved with BL21 strain co-transformed with the plasmid pET21-*nfs1* and pSBET. The *E. coli* BL21 cells transformed with the different expression vectors were grown at 37 °C in Luria-Bertani medium supplemented with the appropriate antibiotics. When A_{600} reached 0.6, 1 mM isopropylthio-β-D-galactoside was added to induce recombinant protein synthesis. The cells were further grown for 16 h at 28 °C, harvested, and centrifuged for 20 min at 4,000 × *g*. After resuspension in appropriate buffer, each bacterial pellet was disrupted by sonication with a Vibra-Cell disrupter (100 pulses every 3 s on power setting 5). The soluble protein extract was separated from the cell debris by centrifugation at 18,000 × *g* for 30 min.

Purification of Recombinant Bio2 and Adx1 Proteins—Bio2 and Adx1 proteins were both purified by metal-chelate column chromatography. The cells containing the overexpressed Bio2 or Adx1 proteins were resuspended in buffer D (20 mM Tris-HCl (pH 7.9), 500 mM NaCl)) supplemented with 5 mM imidazole. The soluble protein extract was applied to a nickel-nitrilotriacetic acid-agarose column (1.6 × 4 cm, Qiagen) previously equilibrated with 30 ml of buffer D supplemented with 5 mM imidazole. The column was washed with buffer D supple-

TABLE I
Engineering of expression vectors

Full-length cDNAs (*adxR, adx1, adx2,* and *nfs1*) and truncated cDNAs (*adx1m,* encoding the putative mature Adx1 protein (amino acids 44-197)) were obtained by PCR amplification using the appropriate primers. The name and the primer sequences used are indicated. An *Nco*I or *Nde*I restriction site (underlined) was introduced in each 5′-primer. The full-length PCR fragments were cloned into the plasmid pPCR-Script (*nfs1* and *adxR*) or pCR4Blunt-TOPO (adx1, adx1m, and adx2). Restriction enzyme couples used to excise the DNA fragments containing the coding sequences are reported. The resulting DNA fragments were then subcloned onto pET29 (*adxR*), pET28 (*adx1m* and *adx2*), and pET21 (*nfs1*) expression vectors.

Primer name	Primer sequence (5′-3′)	Digestion	Expression vector construction
AdxR cDNA cloning			
5′-AdxR	GGTCTTTGAAATTGGTGACCATGGGTAGATATCTAGC	*Nco*I-*Hind*III	pET29-*adxR*
3′-AdxR	GGCCCTAGTTGGCTGCTGCTGCTAATAGATCC		
Adx1 cDNA cloning			
5′-Adx1	CGGCGCCAAACATATGGTCTTCCATAGGC		pET28-*adx1 m*
5′-Adx1 m	GCTCTTTCATCTCGACAGCATATGCTGCAAAGGC	*Nde*I-*Eco*RI	
3′-Adx1	GCTCTGAGACTATTAAGTGACTAGTGAGG		
Adx2 cDNA cloning			
5′-Adx2	GCGGCAGTGACATATGATCGGTCATAGG	*Nde*I-*Not*I	pET28-*adx2*
3′-Adx2	CGAAGAATCTCTGCGGAAACTAATGAGG		
Nfs1 cDNA cloning			
5′-Nfs1	CAAACCGAGGTTTTAGACATATGGCGTCTAAGG	*Nde*I-*Not*I	pET21-*nfs1*
3′-Nfs1	CACAGTTTGAGTGGAACTGTTG		

mented with 50 mM imidazole. The recombinant protein was then eluted using buffer D supplemented with 250 mM imidazole. Fractions containing the recombinant protein (Bio2 or Adx1) were pooled and dialyzed against 50 mM Tris-HCl (pH 8.0), 15% (w/v) glycerol. Purified enzymes were aliquoted and stored at −80 °C until use.

Purification of Recombinant AdxR Protein—Purified Adx1 protein (20 mg) was immobilized on Affi-Gel 10 (Bio-Rad) mini-columns according to the manufacturer's instructions. The cells containing the overexpressed AdxR were resuspended in buffer B and disrupted by sonication. The soluble protein extract (400 mg proteins) was loaded onto a Green-A-matrix (1.6 × 10 cm, Sigma) column equilibrated previously with buffer B. The column was washed with 100 ml of buffer B. Elution was then performed with 50 ml of buffer B supplemented with 1.5 M of KCl (flow rate 0.5 ml/min). Fractions containing proteins were combined, concentrated with Jumbosep-10 tubes, desalted, and loaded (50 mg of proteins) onto the Affi-Gel-Adx1 (1 × 3 cm) column that had been equilibrated with buffer B. The column was washed with 50 ml of buffer B containing 50 mM of KCl. Proteins were eluted with 4 ml of buffer B supplemented with 1 M of KCl. The AdxR-containing fractions were combined, concentrated, and stored aliquoted at −80 °C.

Purification of Recombinant Nfs1 Protein—The overexpressed Nfs1 protein was insoluble in *E. coli*. Inclusion bodies containing the recombinant protein were prepared and purified as described in Ref. 27. After separation on 12% SDS-PAGE, inclusion bodies proteins were stained with Coomassie Blue to locate the Nfs1 protein band. This band was then excised, placed into dialysis tubing (SnakeSkinT Dialysis Tubing 10K, Pierce), submitted to electroelution for 4 h at 50 V in 50 mM NH_4HCO_3, 0.1% SDS, and freeze-dried overnight. The prepared polypeptide (0.5 mg) was resuspended in phosphate-buffered saline and used to obtain rabbit antibodies.

Enzyme Activity Assays—Biotin synthase activity was measured in the presence of the *bio2* gene product (10 μM monomer) and protein fractions (matrix proteins and/or purified recombinant accessory proteins), according to the radiochemical and microbiological protocols described previously (21). The standard reaction mixture, in a final volume of 100 μl, contained 50 mM Tris-HCl (pH 8.0), 2 mM dithiothreitol, 0.5 mM $Fe(NH_4)_2(SO4)_2$, 1 mM NADPH, 0.2 mM AdoMet, 5 mM fructose 1,6-bisphosphate, 0.1 mM thiamine pyrophosphate, 0.15 mM DTB, and 0.5 mM L-cysteine (or 1 mM Na_2S when indicated).

Cytochrome *c* reduction was assayed in 50 mM Tris-HCl (pH 8.0) in the presence of 50 μM cytochrome *c*, 50 μM NADPH, 10 μM Adx1, and 60 nM AdxR. The reduction of cytochrome *c* was followed by the absorption increase at 550 nm.

Electrophoresis and Immunoblotting—SDS-PAGE was performed in slab gels containing 12% (w/v) acrylamide. The conditions for gel preparation, sample solubilization, electrophoresis, and gel staining were detailed by Chua (28). Polypeptides were also transferred electrophoretically onto nitrocellulose sheets (Bio-Rad) as described by Towbin *et al.* (29). For Western blot detections, rabbit antiserum raised against the recombinant mitochondrial Nfs1 protein from *Arabidopsis* and guinea pig antiserum raised against the recombinant AdxR from *Arabidopsis* were prepared (Elevage Scientifique des Dombes, Romans, France). Polyclonal anti-rabbit serum (Bio-Rad) and polyclonal anti-guinea pig serum (Sigma) were used as a second antibody. The presence of immune complexes was analyzed by chemiluminescence with an ECL kit (Roche Applied Science).

Analytical Methods—Protein concentration was determined either by the Bradford method (30) using Bio-Rad protein-assay reagent, with γ-globulin as a standard, or for pure proteins by measuring the absorbance at 205 nm (31). UV-visible spectra were recorded using a Uvikon 860 (Kontron) spectrophotometer. DNA sequencing was performed on both strands using the Prism Kit with fluorescent dideoxynucleotides (Applied Biosystems, Genome Express, Meylan, France). Internal peptide sequencing was performed by Edman degradation after tryptic digestion of the proteins and purification of the resulting peptides by high pressure liquid chromatography (Institut Pasteur, Laboratoire de microséquençage des protéines, Paris, France). Kinetic data were fitted to the appropriate rate equations by nonlinear regression analyses using the Kaleidagraph program (Synergy Software, Reading, PA).

RESULTS

Investigation of the Proteins Required for Biotin Synthase Activity in Vitro—The *bio2* gene product is not able to support biotin synthesis by itself. In a previous study, we have shown that mitochondrial matrix prepared from potato tubers was essential in addition to the *bio2* gene product to synthesize biotin in an *in vitro* reconstituted system (21). In order to identify the accessory proteins required for the reaction in plants, we carried out the fractionation of mitochondrial matrix proteins by different chromatographic steps. First of all, the matrix was fractionated on a gel filtration column (Fig. 1). Four proteinaceous pools were arbitrarily defined and named pool I, II, III and IV. Then 0.5 mg of protein from each of these pools was combined with the purified *bio2* gene product, and the biotin synthase activity of the resulting associations was measured. As shown in Fig. 1*B*, pool IV was the only one efficient in the restoration of biotin synthase activity. However, under our assay conditions, subsequent addition of pool II to Bio2 and pool IV led to a 1.5–2-fold increase of the activity. Therefore, we suppose that pool IV contains all the components involved in the physiological reduction system necessary for biotin synthase activity. Pool II should contain other stimulating component(s) for the biotin synthase reaction.

Identification of the Active Proteins in Pool IV—Pool IV was fractionated further on a DEAE-Sepharose column. None of the individual fractions eluted from the DEAE-Sepharose column were active in the biotin synthase assay. This implied that more than one component was present in pool IV and that these components had been separated on the anion-exchange col-

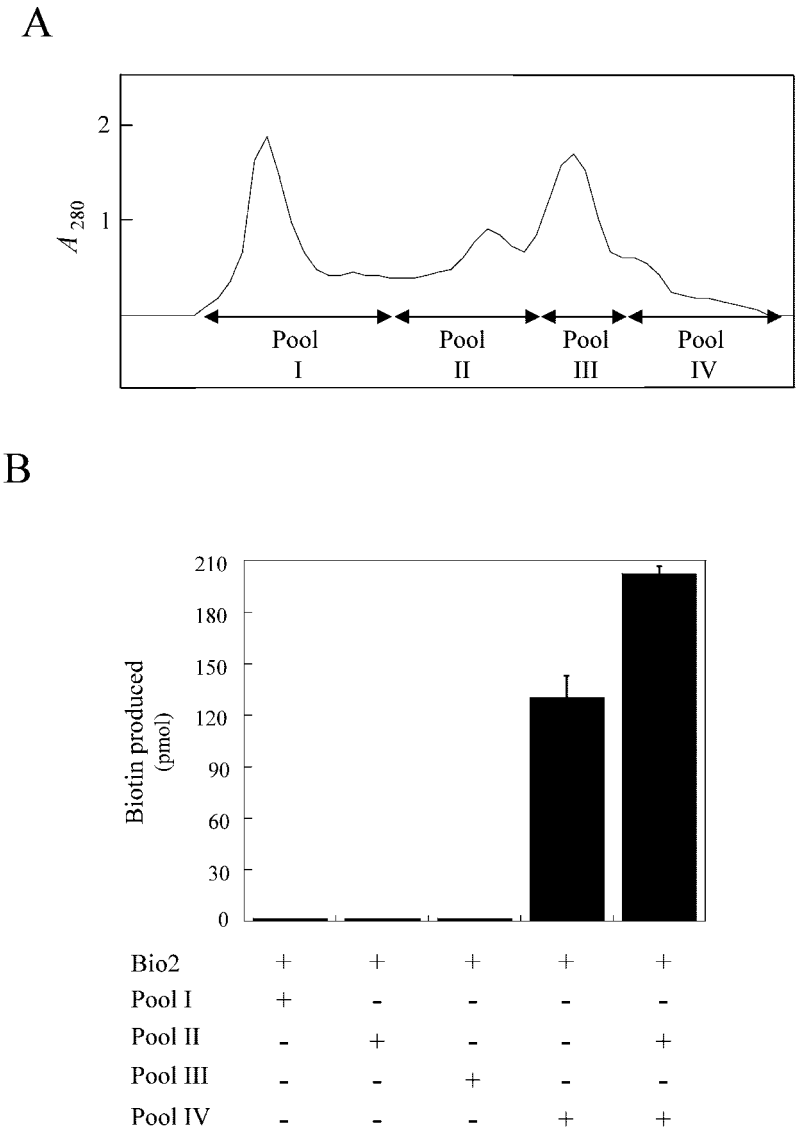
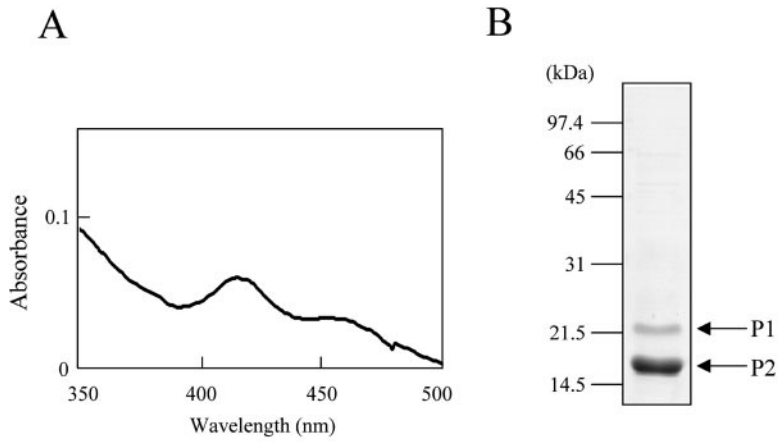


FIG. 1. **Gel filtration chromatography.** *A*, mitochondrial matrix (400 mg of proteins) was applied onto a gel filtration column (Superdex S200), and proteins were eluted as described under "Experimental Procedures." Four proteinaceous pools were arbitrarily defined. *Horizontal arrows* indicate the elution positions of the pools. *B*, aliquots of each pool (0.5 mg of protein) were associated with the *bio2* gene product (10 μM), and biotin synthase activity was measured. Biotin produced during the reaction (2 h at 37 °C) was determined using the microbiological method with *L. plantarum* (see "Experimental Procedures"). The *error bars* represent standard deviations from three independent measurements.

umn. Indeed, under our *in vitro* assay conditions (see "Experimental Procedures"), both fractions eluted with 80–150 and 350–450 mM KCl, respectively, were required, in addition to the *bio2* gene product and the gel filtration pool II (see Fig. 1), to trigger efficient biotin synthase activity.

Identification of the Active Protein in the DEAE Column 350–450 mM KCl Eluted Pool—The DEAE 350–450 mM KCl eluted pool was loaded onto a hydroxylapatite (HTP) column. The active component was eluted with a linear phosphate-buffered gradient. Biotin synthase activity was measured with each of these HTP fractions in association with Bio2, gel filtration pool II (see Fig. 1), and the DEAE 80–150 mM KCl eluted pool. Biotin production was observed with the HTP fractions eluted with 30–50 mM phosphate buffer. At this stage of the purification, a slight red coloration was associated with this mitochondrial active fraction. UV-visible absorbance spectrum showed characteristic peaks for iron-sulfur proteins containing [2Fe-2S] clusters (Fig. 2*A*). SDS-PAGE analysis of the concentrated active fraction revealed the presence of two major proteins, named P1 and P2, and less abundant proteins of higher molecular masses (Fig. 2*B*). The apparent molecular masses of the P1 and P2 proteins were 22 and 16 kDa, respectively. SDS-PAGE analysis of the individual active HTP fractions showed that only the quantitative variation of the P1 protein was perfectly correlated with the quantitative variation of the biotin synthase activity (data not shown). As a result, despite the occurrence of two major proteins, P1 protein and not P2 protein seemed to play a role in the biotin synthase reaction. To confirm this, both P1 and P2 proteins prepared from potato tuber mitochondria were digested with trypsin, and the resulting peptides were purified by high pressure liquid chromatography. Two peptides from P1 protein and one from P2 protein were sequenced, respectively (Fig. 2*C*). As shown in Fig. 2*C*, P2 protein was identified as H-protein, one of the components of the mitochondrial glycine cleavage system by a FASTA search in the *Arabidopsis* genome data base. Thus, as expected, this protein was unlikely to be involved in the physiological reduction system required for biotin synthase activity. On the contrary, a strong similarity was found between the two peptide sequences of the P1 protein and two adrenodoxin-like proteins of *Arabidopsis*, annotated as putative mitochondrial ferredoxins (Fig. 2*C*). Adrenodoxins are small iron-sulfur proteins with electron transfer properties that belong to the large family of the [2Fe-2S]-type ferredoxins ubiquitously found in plants, animals, and bacteria. These properties of Adx made P1 protein a good candidate for being the HTP 30–50 mM phosphate eluted pool component involved in the biotin synthase reaction. It is also responsible for the reddish coloration of this HTP active fraction (Fig. 2*A*). We have isolated and sequenced full-length cDNAs from *Arabidopsis* that we named *adx1* and *adx2*, cor-



C

Protein number	Internal peptides sequences	TAIR FASTA results	MIPS accession number
P1	QTISVTFVDK LGCQVIAKPELDGIR	Putative mitochondrial ferredoxin proteins	At4g21090 At4g05450
P2	KALMDPDQYTK	Glycine cleavage system H protein, mitochondrial precursor	At1g32470

FIG. 2. **Hydroxylapatite active fraction analysis.** Biotin synthase activating fraction eluted by HTP column chromatography was analyzed by UV-visible spectrophotometry (*A*), SDS-PAGE (*B*), and internal peptide sequencing (*C*). *A*, the absorption spectrum was recorded on a Uvikon 860 spectrophotometer with 80 μg of protein from the active HTP fraction. *B*, SDS-PAGE analysis of the HTP-active fraction (15 μg of protein). SDS-PAGE was carried out on a 12% acrylamide gel that was stained with Coomassie Blue. *C*, identification of P1 and P2 proteins by internal peptide sequencing. TAIR FASTA search was performed (www.arabidopsis.org) using internal peptide sequences generated by trypsin hydrolysis of the proteins. The identification results (protein name and MIPS accession numbers (mips.gsf.de/proj/thal/)) are reported.

responding to these putative mitochondrial ferredoxins, as described under "Experimental Procedures." Both Adx1 and Adx2 deduced amino acid sequences consist of 197 residues yielding proteins of 22 kDa. The alignment of Adx sequences from very distant species like *Arabidopsis*, *Bos taurus,* and *Rickettsia prowazekii* shows high overall similarity (about 50%) and high conservation in several protein domains (Fig. 3*A*). Moreover, we evidenced 90% of similarity between both *Arabidopsis* Adx-like protein sequences (Fig. 3*A*). As a result, we realized all subsequent experiments with only one *Arabidopsis* Adx-like protein, the Adx1 protein. The coding sequence for the putative Adx1 mature protein (amino acid 44–197) was cloned into the *E. coli* expression vector pET28 in-frame with an NH_2-terminal His_6 tag coding sequence. Efficient expression of the recombinant protein was achieved in *E. coli* BL21 (DE3) strain, which contains the T7 polymerase system. Then the Adx1 protein was purified by standard nickel-affinity chromatography. On SDS-PAGE, the purified Adx1 protein showed a single band with a molecular mass of ~22 kDa (Fig. 4*A*, *lane 1*). The pure native protein has a reddish coloration and contains a [2Fe-2S] center according to the UV-visible spectrum (Fig. 4*B*). Moreover, the potato HTP biotin synthase activating fraction, resulting from the DEAE 350–450 mM KCl eluted pool fractionation, could be efficiently replaced by this purified recombinant Adx1 protein for *in vitro* biotin synthase reaction (in an assay mixture also comprising Bio2, gel filtration pool II, and the DEAE 80–150 mM KCl eluted pool). All these results indicate that the Adx1 protein is one of the elements involved in the physiological reduction system required for plant biotin synthase activity.

Identification of the Active Protein in the DEAE Column 80–150 mM KCl Eluted Pool—The primary function of Adx in mammals is to supply electrons from an NADPH-dependent adrenodoxin reductase to different electron acceptors. Thus, we speculated that, by inference, an AdxR-like protein was the active component present in the DEAE 80–150 mM KCl eluted pool. Interestingly, a BLAST search in the *Arabidopsis* data base allowed us to identify a protein presenting high overall similarity with the well characterized bovine AdxR (Munich Information Center for Protein Sequences (MIP) accession number At4g32360, annotated ferredoxin-$NADP^+$ reductase like-protein) (Fig. 3*B*). The full-length cDNA from *Arabidopsis* encoding this putative AdxR was isolated and cloned into the *E. coli* expression vector pET29. The deduced amino acid sequence consists of 483 residues yielding a protein of 53 kDa. A two-step protocol was devised to purify to near homogeneity the putative AdxR from *Arabidopsis* overexpressed in *E. coli* cells, including an affinity chromatography onto an Affi-Gel-Adx1 column (for details, see "Experimental Procedures"; Fig. 4*A*, *lane 2*). The pure protein has a yellow coloration, and its UV-visible absorbance spectrum shows characteristic peaks for FAD-containing flavoproteins (Fig. 4*C*). Antibodies raised against this purified *Arabidopsis* putative AdxR were prepared and used for Western blot analysis. As shown in Fig. 5, antibodies raised against the recombinant protein from *Arabidopsis* reacted with a single 50-kDa polypeptide, exclusively in pool IV from the gel filtration chromatography and in the 80–150 mM KCl eluted pool from the DEAE column. More importantly, the substitution of this last fraction by the purified putative AdxR from *Arabidopsis* was efficient for the biotin synthase assay. These results confirm the presence of an AdxR-like protein in the DEAE 80–150 mM KCl eluted pool and strongly suggest that AdxR is associated with Adx1 to form the physiological reduction system required for plant biotin synthase reaction.

Characterization of the Arabidopsis Adx-AdxR Reaction—The efficiency of the electron transfer between recombinant AdxR and recombinant Adx1 from *Arabidopsis* was tested by measuring the cytochrome *c* reduction at 550 nm. This reduction was only observed in the presence of purified AdxR, puri-

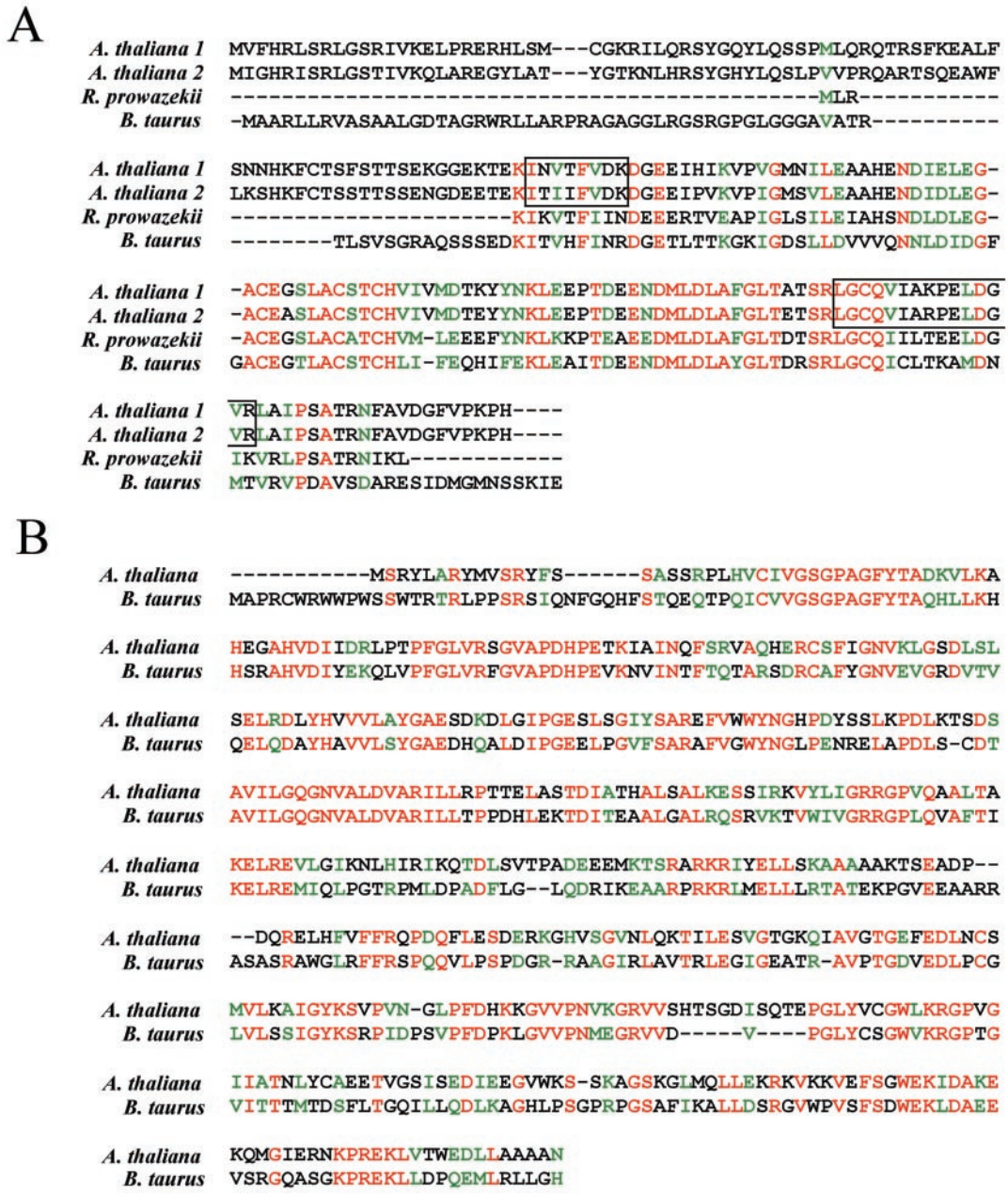


FIG. 3. **Amino acid sequence alignments of adrenodoxins and adrenodoxin reductases from various origins.** Comparison of the primary structures of Adx (*A*) (GenBank™ accession numbers are as follows: *A. thaliana* 1 and 2, AY074925 and AY074926, respectively; *R. prowazekii*, A71731; and *B. taurus*, BAA00363), and AdxR (*B*) (GenBank™ accession numbers are as follows: *A. thaliana*, AY074927, and *B. taurus*, BAA11921). The alignments were generated with the ClustalW program (www.expasy.ch). Identical amino acids are colored in *red* and similar amino acids in *green*. *Arabidopsis* Adx regions showing sequence similarities with the two tryptic peptides of the P1 protein described in Fig. 2 are *boxed*.

fied Adx1, and NADPH. It was completely abolished if one of these three components was absent (data not shown). Consequently, NADPH, AdxR, and Adx1 form an active electron transfer chain allowing the cytochrome *c* reduction. We determined the Michaelis constant of AdxR with NADPH and NADH using the same cytochrome *c* reduction test. The K_m values for NADPH and NADH were 2.4 and 1000 μM, respectively. Moreover, the concentration dependence of Adx1 was explored using NADPH as an electron source. The half-maximal velocity of the reaction was observed with 0.5 μM Adx1. These values are similar to those reported for mammalian proteins (32–34). As a result, the *Arabidopsis* AdxR, like the mammalian proteins, primarily transfers electrons from NADPH. On the contrary, the yeast AdxR equivalent (Arh1p) could receive electrons from either NADPH or NADH because the apparent K_m value is roughly the same for both substrates *in vitro* (35).

Identification of Pool II Component from the Gel Filtration Chromatography Required for the Plant Biotin Synthase Reaction—As described above, AdxR and Adx1 from *Arabidopsis* formed a functional electron transfer chain. However, when biotin synthase activity driven by Bio2 was measured in the presence of these two purified recombinant proteins, biotin production during the reaction was low. Addition of the gel filtration pool II strongly enhanced the biotin production level (Fig. 6*A*). In order to identify the component responsible for this positive effect, gel filtration pool II was fractionated further on a DEAE-Sepharose column. Each of the eluted fractions was tested in the biotin synthase assay (including purified recombinant Bio2, Adx1, and AdxR proteins). Enhanced biotin

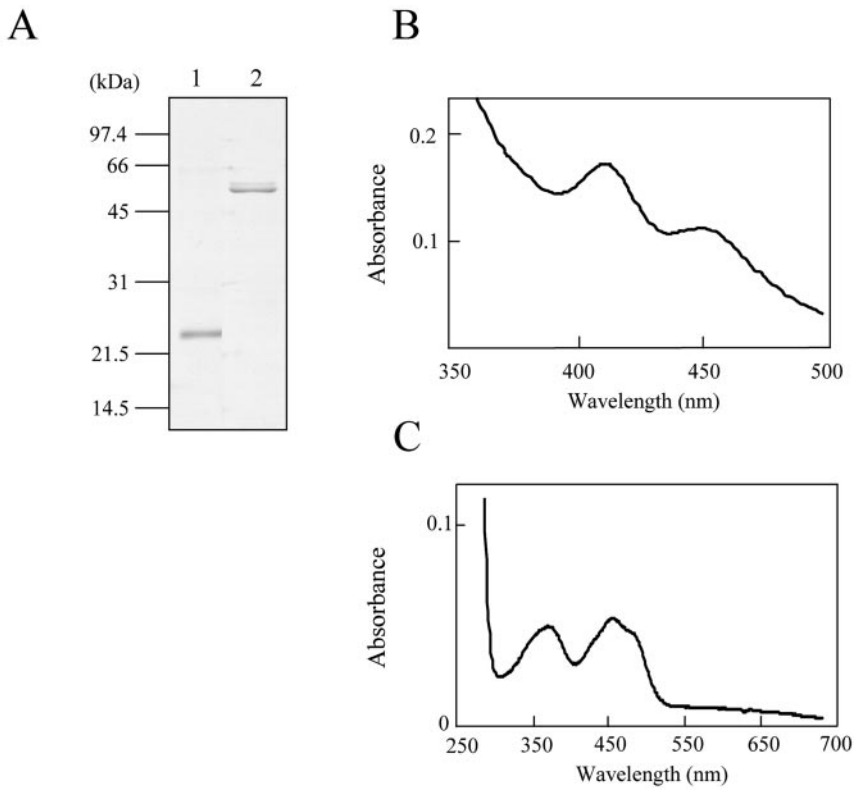


FIG. 4. **Purification and absorption spectra of recombinant Adx1 and AdxR from *Arabidopsis*.** Recombinant Adx1 and AdxR were purified as described under "Experimental Procedures." *A*, purified Adx1 protein (*lane 1*, 1.5 μg) and purified AdxR protein (*lane 2*, 1.5 μg) were analyzed by SDS-PAGE. SDS-PAGE was carried out on a 12% acrylamide gel that was stained with Coomassie Blue. *B*, UV-visible spectrum of purified recombinant Adx1 (125 μg of protein) in 50 mM Tris-HCl (pH 8.0), 15% (w/v) glycerol. *C*, UV-visible spectrum of purified recombinant AdxR (300 μg of protein) in 50 mM Tris-HCl (pH 8.0), 15% (w/v) glycerol.

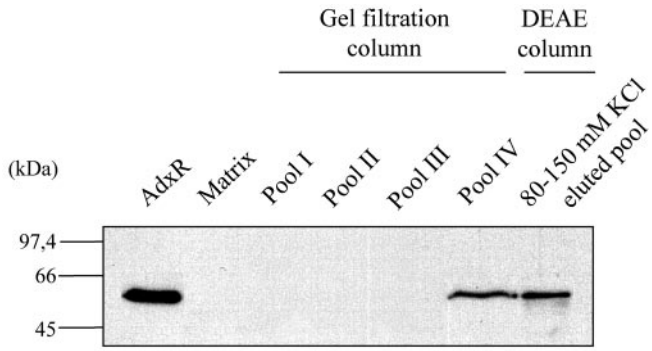


FIG. 5. **Western blot analysis of potato mitochondrial fractions using polyclonal antibodies raised against recombinant adrenodoxin reductase protein from *Arabidopsis*.** Proteins were separated by 12% SDS-PAGE and subjected to Western blot analysis. *AdxR*, 50 ng of purified recombinant AdxR. *Matrix*, 100 μg of mitochondrial matrix proteins. *Pool I–IV*, 100 μg of protein of each gel filtration pool. *80–150 mM KCl eluted pool*, 10 μg of protein of DEAE-Sepharose protein pool eluted with 80–150 mM KCl. The blot was probed with an *Arabidopsis* AdxR polyclonal antibody (dilution to 1:2000). Position of molecular markers, in kDa, is given on the *left*.

production was only observed with the DEAE fractions eluted with 220–280 mM KCl. Interestingly, this protein fraction and cysteine could efficiently be replaced by Na_2S (Fig. 6*A*). As a result, we supposed that the DEAE column 220–280 mM KCl eluted pool probably metabolized the cysteine present in the assay medium for sulfide production. Such proteins, which are able to pull out a sulfur atom from cysteine, have already been described and named cysteine desulfurase. We have cloned a cDNA from *Arabidopsis* encoding a cysteine desulfurase-like protein (Nfs1) (21). The deduced amino acid sequence consists of 453 residues yielding a protein of 50 kDa. Moreover, transient expression of green fluorescent protein fusion protein into *Arabidopsis* cells demonstrated a mitochondrial location for this protein (36). The resulting protein was overexpressed in *E. coli* cells. Unfortunately, the plant recombinant protein was exclusively insoluble in *E. coli* under all the culture conditions tested and was recovered in the inclusion body fraction. Expression of the mature protein devoid of the transit peptide did not improve solubility (data not shown). Nevertheless, the inclusion bodies were purified, and rabbit antibodies raised against this protein were prepared. Then potato mitochondrial matrix, gel filtration pool II, and DEAE column 220–280 mM KCl eluted pool were subjected to Western blot analysis (Fig. 6*B*). Antibodies raised against Nfs1 from *Arabidopsis* reacted with a single polypeptide of 47 kDa in each of these fractions. Moreover, a significant enrichment of the Nfs1-like protein was observed in the DEAE column 220–280 mM KCl eluted pool. In addition, this protein enrichment was perfectly correlated with the biotin synthase stimulating activity. Although no pure soluble recombinant cysteine desulfurase could be tested for biotin synthase stimulating activity in this study, all together our results suggest an essential role of this protein in the biotin synthase reaction.

Reconstitution of Arabidopsis Biotin Synthase System in Vitro—On the basis of the data presented above, we have reconstituted a functional *Arabidopsis* biotin synthase complex *in vitro* and defined new optimal assay conditions with each of the purified accessory proteins identified here. As noted previously, recombinant Nfs1 protein was insoluble in *E. coli* cells. Because Na_2S replaced efficiently the Nfs1-enriched fraction plus cysteine, as a result, Na_2S was subsequently used for all optimization experiments. The concentration dependence of Adx1 and AdxR was first explored. In the presence of 2.5 μM AdxR, biotin production evolves with an increasing concentration of Adx1 and finally saturates at about 5 μM (Fig. 7*A*). In the presence of saturating Adx1, about 1 μM AdxR was required for biotin production (Fig. 7*B*). These values are of the same order of magnitude as those reported for flavodoxin and flavodoxin reductase, which are components of electron transfer chain involved in the *E. coli* biotin synthase reaction (18). Assay conditions were also optimized with respect to small factors. Notably, fructose 1,6-bisphosphate and thiamine pyro-

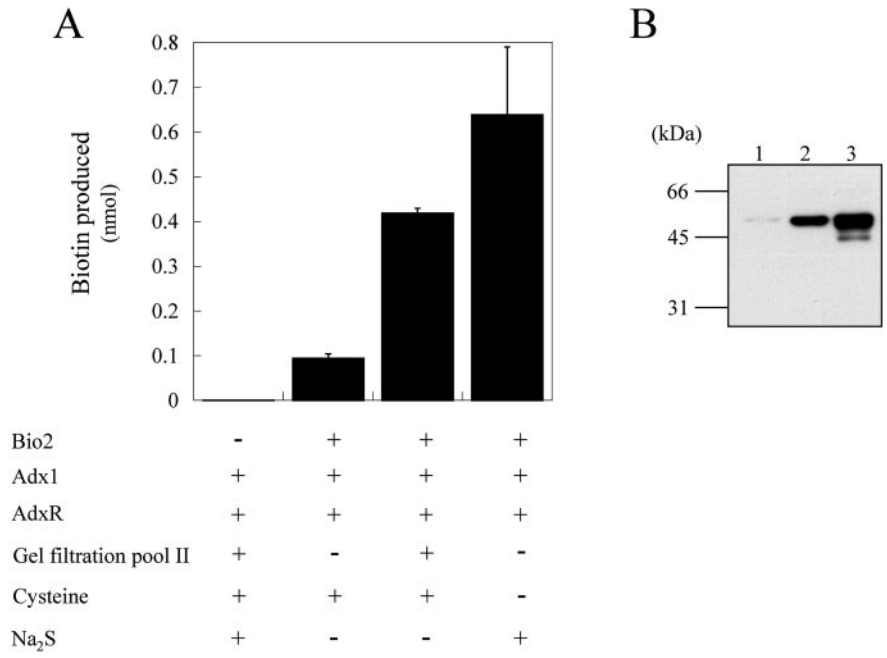


FIG. 6. **Analysis of pool II component required for plant biotin synthase reaction.** *A*, *Bio2* gene product (10 μM), Adx1 (10 μM) and AdxR (2 μM) were associated with or without gel filtration pool II (0.5 mg of protein) (Fig. 1), in the presence of 0.5 mM cysteine or 1 mM Na_2S, and biotin synthase activity was measured. Biotin produced during the reaction (2 h at 37 °C) was determined using the microbiological method with *L. plantarum* (see "Experimental Procedures"). The *error bars* represent standard deviations from three independent measurements. *B*, Western blot analysis of potato mitochondrial fractions using polyclonal antibodies raised against recombinant Nfs1 protein from *Arabidopsis*. Proteins were separated by 12% SDS-PAGE and subjected to Western blot analysis. *Lane 1*, 3 μg of mitochondrial matrix proteins; *lane 2*, 3 μg of gel filtration pool II proteins (see Fig. 1); *lane 3*, 3 μg of gel filtration pool II proteins fractionated on a DEAE-Sepharose column. The blot was probed with an *Arabidopsis* Nfs1 polyclonal antibody (dilution to 1:10,000). Position of molecular markers, in kDa, is given on the *left*.

phosphate, which stimulated biotin synthase activity in a system comprising purified *Arabidopsis* Bio2 protein in combination with unfractionated mitochondrial proteins (21), had no effect on the well defined plant system and thus were subsequently omitted. The reconstitution of an active *Arabidopsis* biotin synthase complex was also checked using the radiochemical method with [^{3}H-DTB] as the source of radioactive label (21). This test has the advantage of making visible the product of the *Arabidopsis* biotin synthase reaction. Data shown in Fig. 8 confirmed that neither recombinant *Arabidopsis* Adx1 nor AdxR alone could support the conversion of DTB to biotin by biotin synthase. However, when AdxR and Adx1 were added to the assay mixture together at optimized concentrations, biotin synthesis was efficiently restored, as shown by the apparition of a product supporting the same migration properties as a purified standard biotin (Fig. 8).

DISCUSSION

The results presented in this work identified Adx and AdxR as essential mitochondrial accessory proteins involved with Bio2 in the plant biotin synthase reaction (Fig. 9). Identification and characterization of these components were achieved by a unique combination of biochemical screening and genomic based approaches. Both *Arabidopsis* cDNAs encoding these proteins were cloned, and the corresponding proteins were expressed in *E. coli* cells and purified. In association with Bio2, Adx and AdxR formed an efficient physiological reduction system, allowing the reconstitution, for the first time, of a functional and well defined *in vitro* plant biotin synthase complex. Even if Adx1 had already been identified by the *Arabidopsis* mitochondrial proteome analysis (37), no function was associated with this protein. Therefore, the *bio2* gene product of *Arabidopsis* is the first identified partner for this specific redox protein in plants. We also report here, to our knowledge, the first biochemical characterization of a plant Adx-AdxR reaction.

Is that the only function of Adx and AdxR in plant mitochondria? Very little is known about these proteins in plants, and the main knowledge comes from studies realized in animals, yeast, and bacteria. In mammals, they are involved in the cytochrome P450 reduction, enabling steroid hormone biosynthesis (38). In yeast, they have an important role in the heme A synthesis, the prosthetic group confined exclusively to mitochondrial and some bacterial cytochrome oxidases (39). Another function of two members of the Adx subfamily, Fdx in *E. coli* and Yah1p in yeast mitochondria, has attracted much attention in recent years. The *fdx* gene, located in the isc (iron-sulfur cluster) operon together with *iscS*, *iscU*, *iscA*, *hscB*, and *hscA*, is crucial for the efficient biosynthesis of Fe-S clusters in *E. coli* (40–42). A similar machinery has been identified in yeast mitochondria (43). The *YAH1* gene is essential for viability of yeast cells, and depletion of Yah1p causes a marked decrease in the activities of mitochondrial and cytosolic Fe-S proteins (44, 45). In plants, it is unlikely that Adx and AdxR, both localized in mitochondria, reduce cytochrome P450. Indeed, despite a large number of cytochrome P450 genes in *Arabidopsis* genome (46), none of these have been characterized in mitochondria (37). On the opposite, we cannot exclude the involvement of this redox chain in the plant Fe/S cluster biosynthesis. Indeed, some proteins showing a high overall similarity with yeast proteins involved in this process were found in the *Arabidopsis* data base and predicted to be mitochondrial (36). Therefore, Adx and AdxR could have a dual function with respect to the *bio2* gene product, a specific function in the biotin synthase reaction and a more general role in its Fe/S cluster biosynthesis (47). Finally, recent studies (48, 49) support the conclusion that both biotin synthase and lipoate synthase are strongly mechanistically related proteins. Consequently, the implication of Adx and AdxR in the mitochondrial lipoate synthase reaction that catalyzes the formation of two C-S bonds from octanoic acid could also be suggested.

The proteins involved in the physiological reduction system for plant biotin synthase reaction identified in this report are different from those described for bacterial systems. Indeed, in *E. coli* this system is composed of flavodoxin and flavodoxin (ferredoxin)-$NADP^+$ reductase (14, 17–19). In a previous study, we showed that heterologous interaction between *Arabidopsis* Bio2 protein and bacterial proteins yielded a functional biotin synthase complex (21). Flavodoxin and ferredoxin (flavodoxin)-$NADP^+$ reductase were supposed to be these bacterial components. However, the Adx-type ferredoxin from *E. coli* (Fdx) was recently described as the kinetically and thermodynamically

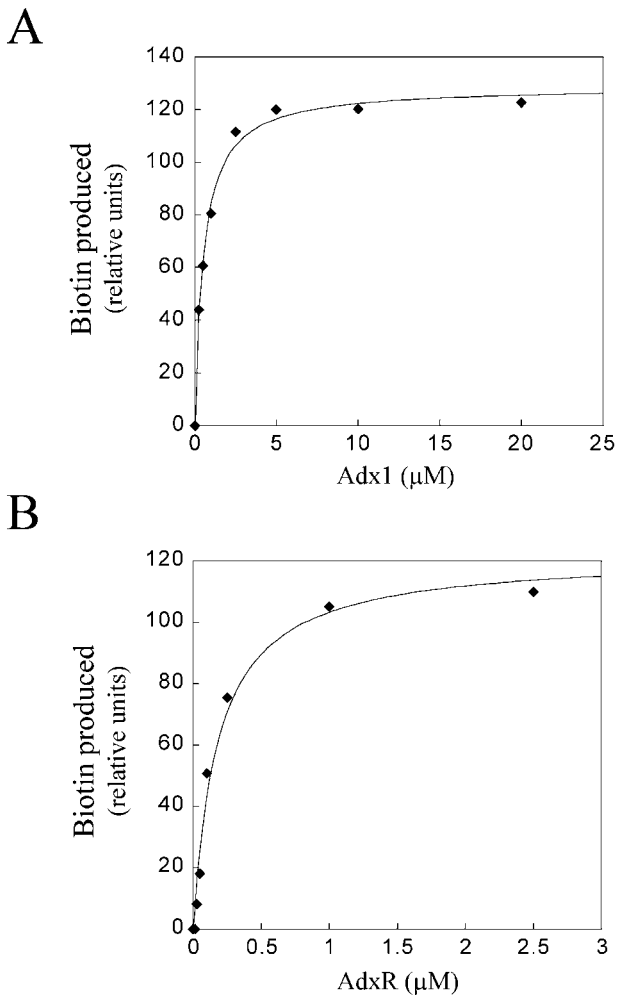


FIG. 7. **Biotin synthase reaction dependence on the adrenodoxin and adrenodoxin reductase concentrations.** Biotin synthase activity was measured under standard conditions (see "Experimental Procedures") by varying the concentration of Adx1 (*A*) or AdxR (*B*) while keeping the other substrates and cofactors at saturating levels. The reaction mixture was incubated for 1 h at 37 °C. Biotin produced during the reaction was determined using the microbiological method with *L. plantarum* (see "Experimental Procedures"). The *lines* represent non-linear regressions to the Michaelis-Menten equation using Kaleidagraph, as described under "Experimental Procedures."

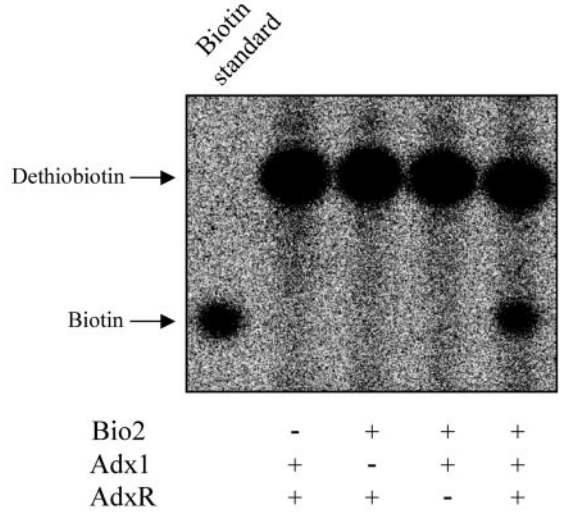


FIG. 8. **Reconstitution of *in vitro* functional *Arabidopsis* biotin synthase complex.** Biotin synthase activity was measured by the conversion of [^{3}H]DTB to [^{3}H]biotin. Substrate and product were separated by thin layer chromatography and detected by PhosphorImaging analysis (21). Different associations between purified recombinant Bio2 (10 μM), Adx1 (10 μM), and AdxR (2 μM) proteins from *Arabidopsis* were tested in the optimized reaction mixture: 50 mM Tris-HCl (pH 8.0), 2 mM dithiothreitol, 0.5 mM $Fe(NH_4)_2(SO_4)_2$, 1 mM NADPH, 0.2 mM AdoMet, 0.15 mM DTB, 1 mM Na_2S. Reaction mixtures were incubated for 2 h at 37 °C. Biotin produced was quantified using ImageQuant software (Amersham Biosciences) and corresponded to 0.5–1 nmol/nmol Bio2 monomer, in the complete assay medium.

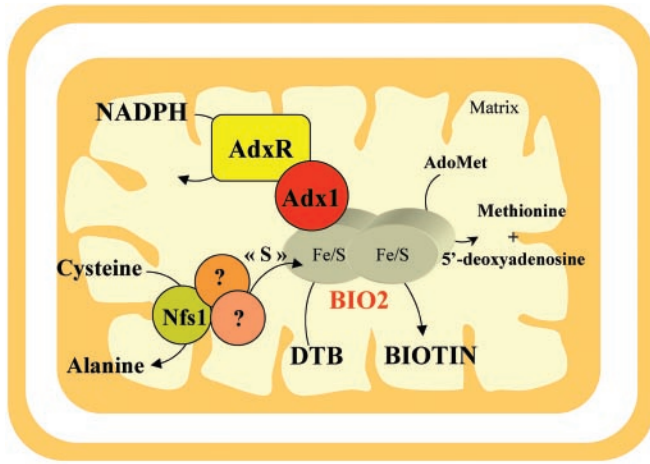


FIG. 9. **Model for the plant biotin synthase reaction in mitochondria.**

preferred partner for ferredoxin (flavodoxin)-$NADP^+$ reductase (50). Consequently, the involvement of this ferredoxin in the *E. coli* biotin synthase reaction is not excluded. Whatever the proteins involved in the electron transfer to Bio2, protein-protein interactions are essential for the formation of an active biotin synthase complex. Further studies, which are in progress in our laboratory, will enable us to elucidate the nature of these interactions. Studies of the mechanism by which these proteins are recruited for biotin synthesis could provide essential information about biotin synthesis regulation in the plant cell mitochondria.

Our results also suggest an essential role of a mitochondrial cysteine desulfurase protein (Nfs1) in the restoration of *Arabidopsis* biotin synthase activity *in vitro*, when cysteine is used as a sulfur donor. This result strongly supports the idea that cysteine is the initial sulfur donor for biotin in plant mitochondria. However, the mechanism by which the sulfur atom is mobilized from cysteine and next transferred into DTB remains unclear. For *E. coli* biotin synthase, two different models are presently proposed. First, the sulfur atom is extracted from cysteine by a cysteine desulfurase. It is next incorporated into DTB via the [2Fe-2S] cluster of biotin synthase, which is the final sulfur donor (10, 15, 51). Second, a pyridoxal 5′-phosphate-dependent cysteine desulfurase activity recently attributed to *E. coli* biotin synthase could provide, via a protein-bound persulfide, the sulfur atom for biotin (52). Amino acids proposed to be involved in the [2Fe-2S] formation by Ugulava *et al.* (10) and in the persulfide binding by Ollagnier-de-Choudens *et al.* (52) are the same. Consequently, the two models are incompatible. In *Arabidopsis*, Nfs1 protein addition led to a marked increase in biotin synthase activity. This confirms and strengthens the results obtained by Kiyasu *et al.* (53) with a crude bacterial cell-free system. Indeed, they have shown that IscS protein (cysteine desulfurase encoded by the *isc* operon) of *E. coli* significantly stimulated the biotin synthase reaction in the presence of cysteine. Interestingly, in the absence of exogenously added cysteine desulfurase, we observe a slight but significant biotin production when the *bio2* gene product is

associated with purified Adx and AdxR with cysteine as sulfur donor (Fig. 6*A*). As a result, we cannot exclude an endogenous pyridoxal 5′-phosphate-dependent cysteine desulfurase activity for the *bio2* gene product. However, contrary to what is observed for the bacterial enzyme (52), pyridoxal 5′-phosphate addition has no effect on *Arabidopsis* biotin synthase activity (data not shown). Finally, as discussed above, plant mitochondria might contain a complex iron-sulfur cluster assembly machinery, including Nfs1, similar to that found previously (36) in yeast. Whether all or part of its components in addition to Nfs1 participates or not in the plant biotin synthase reaction remains to be established (Fig. 9).

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