An FMN-dependent NADH-azoreductase of Escherichia coli was purified and analyzed for identification of the gene responsible for azo reduction by microorganisms. The N-terminal sequence of the azoreductase was consistent with that of the acpD gene product, acyl carrier protein phosphodiesterase. Overexpression of the acpD gene provided the E. coli with a large amount of the 23-kDa protein and more than 800 times higher azoreductase activity. The purified gene product exhibited activity corresponding to that of the native azoreductase. The reaction followed a ping-pong mechanism requiring 2 mol of NADH to reduce 1 mol of methyl red (4′-dimethylaminobenzene-2-carboxylic acid) into 2-aminobenzoic acid and N,N′-dimethyl-p-phenylenediamine. On the other hand, the gene product could not convert holo-acyl carrier protein into the apo form under either in vitro or in vivo conditions. These data indicate that the acpD gene product is not acyl carrier protein phosphodiesterase but an azoreductase.

Azo dyes are widely used for industrial, printing, and clinical purposes as well as textile dyeing because of their chemical stability, ease of synthesis, and versatility. Their durability, however, causes pollution once the dyes are released into the environment as effluent. In addition, some azo dyes are toxic and mutagenic (1).

Azoreductases of microorganisms are favorable for the development of biodegradation systems for such azo dyes, because these enzymes catalyze reductive cleavage of azo groups (-N=N-) under mild conditions. In addition, bacterial enzymes can be readily overproduced in bacterial cells. Identification and overproduction of azoreductase constitute a straightforward approach for the development of biodegradation systems.

Previous studies mainly focused on the screening of microorganisms exhibiting high azoreductase activity. For example, Acetobacter liquefaciens S-1 (2) and Klebsiella pneumoniae RS-13 (3) were reported to exhibit methyl red degradation ability. Two azoreductases were purified from Pseudomonas KS46 (4), and it was revealed that both enzymes utilize NAD(P)H for Orange II (1-(4′-diethylaminoazobenzene-2-carboxylic acid) and N,N′-dimethyl-p-phenylenediamine (DMPD) were from Nacalai Tesque (Kyoto, Japan). Ethyl red (4′-diethylaminobenzene-2-carboxylic acid) and Ponceau SX (3-(2,4-dimethyl-5-sulphophenylazo)-4-hydroxy-1-naphthalenesulfonic acid) were from Tokyo Kasei (Tokyo, Japan). Menadione was from Sigma. NAD(P)/H were from Oriental Yeast (Tokyo, Japan).

Assaying of Azoreductase

The standard assay system for azoreductase comprised 25 mM Tris-HCl (pH 7.4), 25 μM methyl red, 0.1 mM NADH, 20 μM FMN, and 5U of enzyme. The gene product (20 kDa) catalyzed the reduction of azo dyes (Roccellin, Sumifix Black, and Solar Orange) in the presence of NADPH.

On the other hand, acyl carrier protein phosphodiesterase (EC 3.1.4.14) (6) converts holo-acyl carrier protein (ACP) 1 into apoACP by catalyzing hydrolisis of the phosphodiester linkage between Ser36 of ACP and the 4′-phosphopantethein prosthetic group. ACP is a small protein (molecular mass, 8847 Da) that plays a central role in the biosynthesis of fatty acids in bacteria, plant chloroplasts, and other organisms (7, 8). ACP phosphodiesterase is known to be coded by the acpD gene in Escherichia coli. Although the physiological role of ACP phosphodiesterase remains obscure at present (6), its importance has been deduced from the distribution of the gene in other bacteria, such as Azospirillum brasilense, Bacillus halodurans, Bacillus stearothermophilus, Bacillus subtilis, Hae morhobium influenzae, Lactococcus lactis, Mesorhizobium loti, Mycoplasma pneumoniae, Pseudomonas aeruginosa, and Streptomyces coelicolor (16).

In this paper, we report the 4500-fold purification and N-terminal sequencing of a novel azoreductase of E. coli. A search of the translated data bases allowed identification of the acpD gene as the gene for the azoreductase. Overexpression of the acpD gene allowed the preparation of azoreductase, in 190-mg quantities, having no ACP phosphodiesterase activity. The biochemical properties of the azoreductase have also been revealed.

EXPERIMENTAL PROCEDURES

Materials

E. coli JM109, Q-Sepharose FF, Q-Sepharose HP, Blue-Sepharose, Sephacryl S200, phenyl-Sepharose, and a Superdex 200 HR 10/30 pre-pack column were from Amersham Pharmacia Biotech. GIGAPITE was from Seikagaku (Tokyo, Japan). Methyl red, FAD, FMN, 2-aminobenzoic acid (ABA), and N,N′-dimethyl-p-phenylenediamine (DMPD) were from Nacalai Tesque (Kyoto, Japan). Ethyl red (4′-diethylaminobenzene-2-carboxylic acid) and Ponceau SX (3-(2,4-dimethyl-5-sulphophenylazo)-4-hydroxy-1-naphthalenesulfonic acid) were from Tokyo Kasei (Tokyo, Japan). Menadione was from Sigma. NAD(P)/H were from Oriental Yeast (Tokyo, Japan).

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enzyme, in a final volume of 2 ml. The reaction was initiated by addition of the enzyme. The initial reaction rate was determined by monitoring the decrease in absorbance at 430 nm in the first 1.5 min in a glass cuvette of 1.0-cm light path at 30 °C. The enzyme activity was a linear function of the incubation time. One unit of methyl red reductase activity was defined as the amount catalyzing the degradation of 1 μmol of methyl red/min at 30 °C using a molar absorption coefficient of 23,360 M⁻¹ cm⁻¹. When methyl red was replaced with other azo dyes, the following molar absorption coefficients and wavelengths were used: 21,560 M⁻¹ cm⁻¹ (ethyl red at 450 nm) and 18,680 M⁻¹ cm⁻¹ (Ponceau SX at 500 nm). We confirmed that the absorption of the azo dyes. One unit of menadione reductase activity was defined as the amount catalyzing the reduction of 1 μmol NAD(P)H/min at 30 °C.

Expression and Purification of the acpD Gene Product

The acpD gene was obtained by PCR using genomic DNA of E. coli strain JM109 as the template. Pfu turbo DNA polymerase (Stratagene) and oligonucleotide primers (sense, 5'-ggcgtatcagcaactttagct-3' containing an NdeI site; antisense, 5'-ggcgtctcttgccatcacttta-3' containing a XhoI site) were used for PCR. The resulting DNA was cloned into the corresponding restriction site of pET22b to obtain a plasmid designated as pETacpD. The nucleotide sequence of the cloned DNA was confirmed by dyeoxy sequencing. E. coli JM109 (DE3) harboring pETacpD was grown at 37 °C in LB medium. Expression was induced with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) for 3 h with an optical density at 600 nm of 0.6. The recombinant AcpD was purified by Q-Sepharose, Blue-Sepharose, Sephacryl S200, and GIGA-PITE column chromatographies.

Stoichiometry of Azo Reduction

Using the standard assay system (2 ml) with 7.8 ng of the purified acpD gene product (designated as AcpD), the methyl red degradation rate and NADH consumption rate in the first 1.5 min were determined by monitoring the absorbance at 430 and 340 nm, respectively. Because the absorbance of methyl red at 340 nm was a linear function of the absorbance at 430 nm under the given conditions, the absorbance at 340 nm in the standard assay system was corrected by calculation. The molar absorption coefficients were as described above.

Identification of Reaction Products

Methyl red was incubated in 40 ml of the standard assay system for 10 min at 30 °C with the purified AcpD (20 μg). For extraction of ABA, an aliquot (20 μl) of the reaction mixture was extracted with ethyl acetate containing an equal volume of ethyl acetate three times, subsequent to adjusting the pH to 3 with 1 N HCl. The extracts were pooled and evaporated in a rotary evaporator. For DMPD, another aliquot (20 μl) was extracted with an equal volume of n-hexane three times, subsequent to adjusting the pH to 10 with 1 N NaOH. The extracts were pooled and evaporated in a rotary evaporator. Each residue was dissolved in 0.5 ml of acetonitrile. CIDP was diluted 20-fold with acetonitrile and then analyzed (20 μl) with a Shimadzu HPLC system equipped with a model SPD-6AV variable wavelength detector (detection wavelength: 336 nm for ABA; 250 nm for DMPD) and a Jasco CrestPak ODS column (4.6 mm × 150 mm). The mobile phase was composed of 25 mM phosphate and acetate, 5.95 (25 mM phosphate:acetate). The flow rate was 0.8 ml/min.

Overexpression and Purification of E. coli ACP

The gene for ACP was amplified using genomic DNA of E. coli strain JM109 as the template for PCR. The forward primer included an EcoRI site before the start codon: 5'-ctggaatttagctgtatcagcaactttagct-3'. The reverse primer included a HindIII restriction site after the stop codon: 5'-gggatctgctcttgccatcacttta-3'. The PCR product was subcloned into the EcoRI/HindIII site of the pKK223-3 plasmid, and the resulting plasmid was designated as pKKacpP. E. coli JM109 was transformed with pKKacpP and grown at 37 °C with LB medium. After the cultures had been grown to an optical density at 600 nm of 0.6, expression was carried out with 0.5 mM IPTG for 3 h. The purification procedure followed the method of Therisod et al. (18).

In Vitro Assay for ACP Phosphodiesterase Activity

The assay system comprised 50 mM Tris-HCl (pH 8.5), 0.02 mM MnCl₂, 25 mM MgCl₂, 1 mM dithiothreitol, 20 μg of holo/apoACP, and 3.7 μg of purified AcpD, in a final volume of 0.1 ml. After incubation at 35 °C for 12 h, a sample was analyzed by native PAGE (19). For the holoACP standard, 20 μg of holo/apoACP was converted to holoACP under the conditions with 50 mM Tris-HCl (pH 8.8), 0.1 mM CoA, 25 mM MgCl₂, 1 mM DTT, and 4.2 μg of ACP synthase. ACP synthase was expressed and purified by a procedure based on that of Lambalot and Walsh (20). For the apoACP standard, Ser⁵⁰-substituted ACP was used. PCR-based site-directed mutagenesis was carried out with substitution from Ser to Cys. The expression and purification procedure were the same as those for the wild type.

N-terminal Amino Acid Sequencing

One micromolar of the purified enzyme was run on a 12.5% SDS-polyacrylamide ‘Tricine gel (1 mm thick) (17), and then blotted onto a ProBlot™ poly(vinylidene difluoride) membrane (Applied Biosystems). The protein band was examined with an Applied Biosystems model 477A protein sequencer, fitted with an on-line model 120A analyzer for the detection of phenylthiobutyrylaminoc acid. In Vivo Assaying of ACP Phosphodiesterase Activity

ACP was coexpressed with AcpD in E. coli and then analyzed by native PAGE. To obtain a plasmid (designated as pACYCacpP) that was compatible with pETacpD and produced ACP, the BamHI-HincII 0.6-kb DNA fragment of pACYC177 (21) was replaced by the 1.4-kb BamHI-Poul fragment (the cohesive end of the Poul site was blunt-ended) from pKKacpP. The 1.4-kb fragment contained a tac promoter, the ACP gene, and then analyzed by native PAGE. To obtain a plasmid (designated as pACYCacpP) that was compatible with pETacpD and produced ACP, the BamHI-HincII 0.6-kb DNA fragment of pACYC177 (21) was replaced by the 1.4-kb BamHI-Poul fragment (the cohesive end of the Poul site was blunt-ended) from pKKacpP. The 1.4-kb fragment contained a tac promoter, the ACP gene, and then analyzed by native PAGE.
and rrnB ribosomal RNA transcription terminators. *E. coli* JM109 (DE3) was cotransformed with pACYCacP and pETacP or pET21a (for a control experiment). Expression was induced by the addition of IPTG as described above.

**Other Techniques**

Protein concentrations were determined using protein assay reagent (Bio-Rad), with bovine serum albumin as the standard. SDS-PAGE was carried out using 12.5% gels as described by Laemmli (22). The proteins on gels were stained using a Silver Stain Kit II (Wako Pure Chemicals, Osaka, Japan). The native molecular weight of the protein was determined by gel filtration on a Superdex 200 HR column (30 x 1.0 cm) that had been equilibrated with Tris-buffered saline buffer (20 mM Tris-HCl (pH 7.5) and 0.15 M NaCl). Calibration of the column was carried out with the following proteins: alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), and cytochrome c (12.4 kDa), all from Sigma.

**RESULTS**

**Purification of Azoreductase**—The azoreductase activity in the 12,500 x g supernatant of the *E. coli* homogenate was 0.014 units/mg. The enzyme was purified by gel filtration on a Superdex 200 HR column (30 x 1.0 cm) that had been equilibrated with Tris-buffered saline buffer (20 mM Tris-HCl (pH 7.5) and 0.15 M NaCl). Calibration of the column was carried out with the following proteins: alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), and cytochrome c (12.4 kDa), all from Sigma.

The azoreductase exhibited molecular masses of 23 and 42 kDa on SDS-PAGE and gel filtration, respectively.

**Amino Acid Sequence Analysis**—The N terminus of the azoreductase was not blocked and revealed to be NH₂-Ser-Lys-Val-Leu-Val-Leu-Lys-. The sequence conformed to the acpD gene product (AcpD), known as ACP phosphodiesterase (6).

**Identification of AcpD as the Azoreductase**—To confirm the azoreductase activity of AcpD, the acpD gene was overexpressed in *E. coli*. In all purification procedures (Fig. 2 and Table II), AcpD behaved the same as the native azoreductase. Furthermore, AcpD exhibited identical migration with the azoreductase in an SDS-PAGE gel (Fig. 1, lane 8). The enzyme activity was also the same as that of the azoreductase (Table III). These results indicate that the azoreductase is identical with AcpD.

**Spectroscopic Properties of AcpD**—The absorption spectrum of AcpD (0.47 mg/ml) exhibited a single peak at 278 nm, ranging in wavelength from 250 nm to 600 nm. The single peak indicated the absence of a flavin cofactor. The fluorescence emission of AcpD (0.22 mg/ml, 11 μM) showed a maximum at around 310 nm, ranging from 280 to 380 nm (Fig. 3). When the enzyme solution was titrated with FMN or FAD, the fluorescence at 310 nm was gradually quenched. The degree of quenching by 12 μM (slightly more than the stoichiometric amount) FMN and FAD was 70 and 30%, respectively (Fig. 3). The greater quenching by FMN implied the FMN specificity of AcpD.

**Steady State Kinetic Analysis of AcpD**—Double-reciprocal plots of initial velocity versus NADH or methyl red concentration resulted in parallel patterns (Fig. 4, A and B). The patterns are consistent with a ping-pong mechanism. Secondary plots of the intercepts against the reciprocals of variable substrate concentrations gave linear correlations. The *Kₘ* values for NADH and methyl red were calculated to be 31.6 and 17.9 μM, respectively.

**Identification of Reaction Products**—The products of the
The activity is expressed as the mean ± S.E. (n > 3). In parentheses is the relative activity with the FMN-dependent methyl red reductase activity taken as 100%.

| Substrates | Cofactors | Azoreductase | AcpD |
|------------|-----------|--------------|------|
| Methyl red | FMN, NADH | 63.9 ± 1.5 (100) | 188.6 ± 5.2 (100) |
| Methyl red | FAD, NADH | 27.2 ± 1.0 (43) | ND |
| Methyl red | FMN, NADPH | ND | ND |
| Methyl red | None, NADH | ND | ND |
| Methyl red + dicumarol | FMN, NADH | 11.1 ± 0.3 (17) | 31.5 ± 0.5 (17) |
| Ethyl red | FMN, NADH | 131.7 ± 2.3 (206) | 44.10 ± 12.7 (234) |
| Ponceau SX | FMN, NADH | ND | ND |
| Menadione | FMN, NADH | 147.9 ± 2.6 (231) | 362.6 ± 15.6 (192) |

* The concentrations of all substrates were 25 μM.
* Dicumarol was added to a final concentration of 2.2 μM.
* Quinone reductase activity was measured.
* ND, not detected.

Standard reaction of AcpD were extracted under acidic (pH 3) and basic (pH 10) conditions. HPLC analyses of the extracts revealed peaks of ABA (retention time, 2.23–2.25 min) and DMPD (retention time, 4.16–4.23 min). The retention times of ABA and DMPD completely coincided with those of each authentic compound. In the absence of AcpD, NADH, or FMN, no peak other than that of methyl red (retention time, 5.2 min) was observed. Thus, AcpD catalyzed the reductive cleavage of methyl red into ABA and DMPD. Spectrophotometrically, 1 mol of methyl red was reduced with 2 mol of NADH. This coincided with the expected stoichiometry for reductive cleavage of the azo bond of methyl red.

ACP Phosphodiesterase Activity in Vitro—An in vivo assay was also carried out to examine the function of AcpD. Because proteins other than the ACP-derived species were observed to remain in the stacker gel (23), crude AcpD preparations could be analyzed by native PAGE (Fig. 6). The cell extract of E. coli overproducing AcpD (Fig. 6, lane 4) was compared with that of the control E. coli (Fig. 6, lane 4) by native PAGE. AcpD was observed to be exclusively in its functionally active holo form regardless of AcpD overexpression.

**DISCUSSION**

To gain insights into the gene and properties of azoreductase, we purified native azoreductase from the E. coli JM109 strain and identified acpD as the gene encoding the azoreductase. Overexpression of the acpD gene allowed the isolation of more than 190 mg of the gene product (AcpD). AcpD was catalytically similar to the native azoreductase, and exhibited the same molecular weights under denatured and nondenatured conditions as those of the native azoreductase as well as N-terminal sequence identity. Identification of the Reaction Product—HPLC analysis indicated that the decolorization of methyl red by AcpD is followed by cleavage of the molecule into colorless compounds, ABA and DMPD. The results proved that the decolorization of methyl red by AcpD was associated with reductive cleavage of the azo bond.

**Kinetic Properties of AcpD**—The decolorization of methyl red by AcpD was analyzed using double-reciprocal plots of initial velocity versus NADH or methyl red concentration. The parallel lines obtained with the assays suggest that, as with methyl red, the catalysis mode of AcpD is a ping-pong Bi-Bi mecha-
This mechanism suggests that FMN mediates electron-transfer from NADH to methyl red. This catalysis mode is generally observed for oxidoreductases containing a flavin prosthetic group such as quinone oxidoreductase. In view of the quinone (menadione) reductase activity observed for AcpD (Table III), the proposed catalysis mode is feasible. Apparently, AcpD required 2 mol of NADH for the decolorization of 1 mol of methyl red. This stoichiometry implies that two cycles of the ping-pong mechanism were required for the cleavage (Scheme 1). In any case, the expression system reported here will provide a sufficient amount of azoreductase and greatly facilitate mechanistic studies on the reductive cleavage of azo dyes.

Comparison of AcpD with Other Enzymes—Previously, two azoreductases were purified from E. coli K12 (24). These enzymes, 12.5 and 28 kDa on SDS-PAGE, utilized NAD(P)H for Ponceau SX degradation. For AcpD, the molecular mass was 23 kDa on SDS-PAGE (Fig. 1), and NADPH was virtually ineffective as a cofactor. Furthermore, AcpD could not degrade Ponceau SX (Table II). Therefore, AcpD is different from the azoreductases of 12.5 and 28 kDa.

Orange II azoreductase (3) was purified from Pseudomonas KF46. The enzyme exists as a monomer with a molecular mass of 30 kDa, and there is no enzyme-bound FAD. Although this enzyme requires NAD(P)H as an electron donor for its activity, a flavin cofactor is not required. Because AcpD requires FMN for its activity (Table III), AcpD must belong to a different protein family from Orange II azoreductase.

Azoreductase from Bacillus sp. was reported recently (5). The NADPH-dependent azoreductase comprises 178 amino acids and contains an NADH-binding motif (GXXGXG). Although this molecular size is similar to that of AcpD, we could not find this motif or significant homology in AcpD.

NAD(P):quinone acceptor oxidoreductase (NQO1, EC 1.6.99.2) is a FAD-containing enzyme that catalyzes the reduction of azo dyes (25) as well as quinones, and thus sequence comparison between AcpD and NQO1 from rat was performed. NQO1 consists of two separate domains (26): a major, “catalytic domain” (residues 1–220), and a small, “C-terminal domain” (residues 221–273). The catalytic domain is involved in the binding of the FMN moiety of FAD and the nicotinamide ribose of NADP⁺, whereas the C-terminal domain is responsible for the binding of adenine riboses from FAD and NADP⁺ (26). AcpD exhibited moderate homology (Fig. 7) to the “catalytic domain” and highly conserved the residues involved in the binding of nicotinamide ribose in rat NQO1 (26).

Fig. 5. Separation of ACP derivatives by native PAGE. Lane 1, purified ACP (2 µg); lane 2, AcpD-treated ACP (2 µg); lane 3, ACP synthase-treated ACP (2 µg); lane 4, purified ACP mutant S36C (1 µg); lane 5, AcpD (0.37 µg).

Fig. 6. Native PAGE analysis of ACP derivatives contained in the crude extract. Lane 1, purified ACP mutant S36C (1 µg) as the apoACP standard; lane 2, purified ACP (2 µg); lane 3, extract of E. coli JM109 (DE3) harboring pETacpD and pACYCacpP (20 µg); lane 4, extract of E. coli JM109 (DE3) harboring pET22b and pACYCacpP (18 µg).

Fig. 7. Amino acid sequence alignment of AcpD and the catalytic domain (1–220 residues) of rat quinone reductase (NQO1). Black and gray boxes indicate identical and similar amino acids, respectively. The similar amino acid groups are as follows: D and N; E and Q; S and T; K and R; F, Y, and W; L, I, V, and M. Arrowheads indicate the residues involved in the binding of the FMN moiety of FAD in rat NQO1 (26). Circles show the residues involved in the binding of nicotinamide ribose in rat NQO1 (26).
C-terminal domain of NQO1 is lacking in AcpD. These observations corroborate the cofactor specificity of AcpD from a structural aspect and imply that AcpD belongs to the quinone oxidoreductase family.

**ACP Phosphodiesterase Activity—**ACP phosphodiesterase activity of AcpD was not detected in *in vivo* or *in vitro* in this study. On the other hand, phosphodiesterase activity had been detected *in vitro* by monitoring the release of phospho-[3H]pantetheine from labeled ACP in an earlier study (6). This discrepancy could be accounted for by misidentification of ACP phosphodiesterase. Therefore, the protein band of ACP phosphodiesterase was enriched in AcpD because of the lower resolution on electroelution. The purified fraction could be poor in authentic ACP phosphodiesterase but rich in AcpD because of the low resolution on electroelution.

Discrepancies could be accounted for by misidentification of ACP phosphodiesterase. This discrepancy was detected *in vivo* activity of AcpD was not detected by electroelution, because the phosphodiesterase remained acetylated.

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Putative ACP Phosphodiesterase Gene (acpD) Encodes an Azoreductase
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J. Biol. Chem. 2001, 276:46394-46399.
doi: 10.1074/jbc.M104483200 originally published online October 2, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M104483200

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