

## Characterization of a Nitrophenol Reductase from the Phototrophic Bacterium *Rhodobacter capsulatus* E1F1

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**The phototrophic bacterium *Rhodobacter capsulatus* E1F1 photoreduced 2,4-dinitrophenol to 2-amino-4-nitrophenol by a nitrophenol reductase activity which was induced in the presence of nitrophenols and was repressed in ammonium-grown cells. The enzyme was located in the cytosol, required NAD(P)H as an electron donor, and used several nitrophenol derivatives as alternative substrates. The nitrophenol reductase was purified to electrophoretic homogeneity by a simple method. The enzyme was composed of two 27-kDa subunits, was inhibited by metal chelators, mercurial compounds, and  $\text{Cu}^{2+}$ , and contained flavin mononucleotide and possibly nonheme iron as prosthetic groups. Purified enzyme also exhibited NAD(P)H diaphorase activity which used tetrazolium salt as an electron acceptor.**

Nitroaromatic pesticides, herbicides, dyes, and explosives are widely synthesized by chemical industries and have been classified as common pollutants in natural ecosystems (12). These compounds are degraded by soil heterotrophic bacteria, as well as by bacterial consortia, through metabolic pathways, including oxidative and reductive processes (4, 8, 9, 17, 19, 20, 22, 32). Oxidative pathways are catalyzed by oxygenases, which have been well characterized from *Pseudomonas* species (32). Oxidation of nitrotoluene by toluene dioxygenase has been reported recently (24).

Microbial detoxification of nitroaromatic compounds by reduction of nitro groups has been extensively studied at the physiological level (1, 9, 17, 19, 23), but there have been only a few reports concerning molecular characterization of the enzymes involved (13, 28). Nitro groups can be reduced by several biological systems, including (i) illuminated chloroplasts with ascorbate-dichlorophenol indophenol as the reductant (7), (ii)  $\text{H}_2$ -hydrogenase and ferredoxin from *Clostridium pasteurianum* or *Veillonella alcalescens* (7, 20), (iii) NAD(P)H nitroreductases from *Nocardia* sp. (4, 28) and *Bacteroides fragilis* (13), and (iv) extracellular nitroreductases from anaerobic human gastrointestinal bacteria (23). These enzymatic systems reduce nitro groups independent of the position of the nitro groups on the aromatic ring. In addition to these pathways, initial hydrogenation of the aromatic ring of picric acid has been reported in *Rhodococcus erythropolis* (15).

Phototrophic bacteria exhibit limited capacities to degrade aromatic compounds such as benzoates (10) and benzoate derivatives (31). Until now, there has been only one report concerning the metabolization of nitrophenols in a phototrophic bacterium. Strain E1F1 of *Rhodobacter capsulatus* uses 2,4-dinitrophenol (2,4-DNP) as an electron acceptor in the presence of light under anaerobic conditions (1). As this strain is capable of photoassimilating nitrate and nitrite (5), the nitro groups can also be used as a nitrogen source, but only under microaerobic conditions in the presence of light (1). In this work, we studied the enzyme responsible for the photoreduction of 2,4-DNP to 2-amino-4-nitrophenol in *Rhodobacter capsulatus* E1F1. This nitrophenol reductase

activity was purified to electrophoretic homogeneity, and the molecular and regulatory properties of the enzyme are described below.

### MATERIALS AND METHODS

**Organism and growth conditions.** *Rhodobacter capsulatus* E1F1 (a gift from W. G. Zumft, Karlsruhe University, Karlsruhe, Germany) was cultured in RCV medium (30) in the presence of light under either anaerobic or microaerobic conditions with 10 mM acetate as the carbon source and in the presence of 0.1 mM 2,4-DNP, as previously described (1). In some experiments (see below) 6 mM  $\text{NH}_4\text{Cl}$  was used as a nitrogen source. In the absence of fixed nitrogen, dissolved  $\text{N}_2$  (16 mg/liter at 30°C under an air atmosphere) was a suitable nitrogen source for bacterial growth.

Unless otherwise stated, the cells were grown in the presence of light under anaerobic conditions in medium containing 2,4-DNP and no additional nitrogen source at the expense of dissolved dinitrogen.

**Purification procedure.** Cells were harvested by centrifugation at  $15,000 \times g$  for 20 min at the mid-log phase of growth, washed with 0.5 M phosphate buffer (pH 6.8) containing 0.2 mM 2,4-DNP, and resuspended in the same buffer. The washed cells were broken by cavitation at 170 W for 15 s and then centrifuged at  $37,000 \times g$  for 20 min. The supernatant was used as the crude extract.

Subcellular fractions were obtained as previously described (21).

(i) **Ultracentrifugation.** Crude extract was centrifuged at  $100,000 \times g$  for 90 min, and the clear supernatant was used for further purification.

(ii) **Ammonium sulfate fractionation.** Ultracentrifuged supernatant was treated with solid ammonium sulfate (35% saturation), the suspension was centrifuged at  $35,000 \times g$  for 15 min, and the resulting pellet was discarded. The supernatant was adjusted to 70% ammonium sulfate saturation. After 15 min of stirring, the solution was centrifuged at  $35,000 \times g$  for 15 min, and the resulting pellet was resuspended in 50 mM phosphate buffer (pH 6.8) containing 50 mM NaCl and 0.2 mM 2,4-DNP (buffer A).

(iii) **Chromatography.** The 35 to 70% ammonium sulfate fraction was passed through a Sephadex G-75 column (50 by

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2.5 cm) equilibrated with buffer A. Proteins were eluted with the same buffer at a flow rate of 35 ml/h, and the fractions containing nitrophenol reductase activity were pooled.

Pooled fractions from the Sephadex G-75 column were applied to a DEAE-Sephacell column (10 by 2.5 cm) equilibrated with buffer A. The column was washed with buffer A containing 0.1 M NaCl, and nitrophenol reductase was eluted with modified buffer A containing 0.15 M NaCl at a flow rate of 30 ml/h.

(iv) **Preparative electrophoresis.** Nitrophenol reductase was purified to homogeneity by using an ELFE system for preparative electrophoresis. Concentrating and separating gels were prepared as described by Jovin et al. (11). Electrophoresis was performed at 15 mA in 0.36% Tris–1.53% glycine buffer (pH 8.3) containing 0.2 mM 2,4-DNP.

**Enzymatic assays.** Nitrophenol reductase activity was assayed by high-performance liquid chromatography (HPLC). The assay mixture contained (in a final volume of 1 ml) 100  $\mu$ mol 2-(*N*-morpholino)ethanesulfonate (MES)-NaOH (pH 6.0) buffer, 0.2  $\mu$ mol of 2,4-DNP, 0.6  $\mu$ mol of NADPH, and an appropriate amount of enzyme. After 15 min at 30°C, the reaction was stopped by heating the preparation at 100°C for 5 min. After cooling, the sample was centrifuged at 10,000  $\times$  *g* for 5 min, and the supernatant was injected into a reverse-phase HPLC column to determine the amount of 2-amino-4-nitrophenol formed (1). As this enzyme reduced several phenolic nitro derivatives besides 2,4-DNP, we named it nitrophenol reductase.

Diaphorase activity was detected in slab gels by nondenaturing polyacrylamide gel electrophoresis (PAGE) performed as described previously (29). Gels were stained with a solution containing 50 mM MES-NaOH (pH 6.0), 0.6 mM NADPH, and 0.6 mM 2,2'-di-*p*-nitrophenyl-5,5'-diphenyl-3,3'-(3,3'-dimethoxy-4,4'-diphenylene)-ditetrazolium chloride (NBT).

One unit of enzymatic activity was defined as the amount of enzyme activity which catalyzed the appearance of 1  $\mu$ mol of product per min or the disappearance of 1  $\mu$ mol of substrate per min.

**Analytical determinations.** Nitrophenol contents were determined by HPLC as previously described (1).

Flavin content was determined by using a Gold System (Beckman) HPLC chromatograph equipped with a Diode array system which allowed recording of the UV and visible light spectra of the compounds separated into HPLC peaks. A reverse-phase column (15 by 0.4 cm; Spherisorb ODS-2; particle diameter, 5  $\mu$ m; Tracer Analytica) was used. Flavins were eluted by combining linear gradients of pure methanol (from 1 to 50%) and 10 mM phosphate (pH 7.0) (from 99 to 50%) at a flow rate of 1 ml/min. The elution program was completed in 20 min. Samples were prepared by heating at 120°C the enzymatic fractions obtained from preparative electrophoresis. After 20 min of heating, the samples were centrifuged at 20,000  $\times$  *g* for 10 min, and the supernatants were injected into the HPLC column.

Ammonium content was estimated by the microdiffusion procedure (6).

Protein content was estimated as described by Lowry et al. (16), Bradford (2), or Smith et al. (26).

**Determination of molecular parameters.** The molecular mass of the native nitrophenol reductase was obtained from chromatographic and sedimentation data (18). Stokes' radius was calculated as described by Siegel and Monty (25) by using the following standards: bovine pancreas RNase A (1.80 nm), soybean trypsin inhibitor (2.25 nm), bovine erythrocyte carbonic anhydrase (2.43 nm), ovalbumin (2.76 nm),

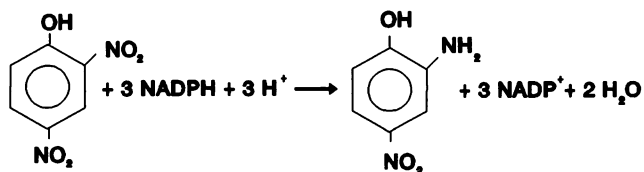


FIG. 1. Stoichiometry of the reaction catalyzed by nitrophenol reductase from *Rhodobacter capsulatus* E1F1.

and bovine serum albumin (3.70 nm). The sedimentation coefficient ( $s_{20,w}$ ) was estimated as described by Martin and Ames (18) by using linear sucrose gradient (5 to 20%) centrifugation of highly purified nitrophenol reductase and the following standards: bovine pancreas RNase A (2.00S), ovalbumin (3.55S), bovine serum albumin (4.31S), yeast alcohol dehydrogenase (7.61S), and bovine liver catalase (11.30S).

The relative frictional quotient ( $f/f_0$ ) was obtained as described by Brewer et al. (3).

The molecular mass of nitrophenol reductase subunits was obtained by sodium dodecyl sulfate (SDS) electrophoresis performed as described by Laemmli (14). The following standards were used: bovine milk  $\alpha$ -lactalbumin (14.2 kDa), soybean trypsin inhibitor (20.1 kDa), phenylmethylsulfonyl fluoride-treated bovine pancreas trypsinogen (24 kDa), bovine erythrocyte carbonic anhydrase (29 kDa), rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (36 kDa), egg albumin (45 kDa), and bovine serum albumin (66 kDa). Proteins in gels were stained with 1% (mass/vol) Coomassie brilliant blue R-250 in 7% (vol/vol) acetic acid. Gels were destained with a mixture containing 10% acetic acid and 10% isopropyl alcohol for 12 h.

**Heme staining.** For the detection of heme groups in slab gels, the heme-staining procedure of Solomonson et al. (27) was used.

**Chemicals.** Flavin standards (flavin mononucleotide [FMN], flavin adenine dinucleotide, and riboflavin), NADH, NADPH, mononitrophenols, MES, protein standards, and Nitro Blue Tetrazolium were purchased from Sigma Chemical Co., St. Louis, Mo. The rest of the nitroaromatic compounds were obtained from Fluka Chemie, Buchs, Switzerland. Sephadex G-75 and DEAE-Sephacell were obtained from Pharmacia, Uppsala, Sweden. All other chemicals were obtained from Panreac, Barcelona, Spain.

## RESULTS AND DISCUSSION

2,4-DNP consumption by *Rhodobacter capsulatus* E1F1 under anaerobic conditions was accompanied by the stoichiometric production of 2-amino-4-nitrophenol, which appeared in the culture medium and was not further reduced (1). The cytosolic fraction of *Rhodobacter capsulatus* E1F1 reduced 2,4-DNP to 2-amino-4-nitrophenol by using NADPH as an electron donor, whereas NADH was 25% less effective. The reaction takes place according to the stoichiometry shown in Fig. 1. This enzymatic activity was very unstable and was absent in both membranes and periplasmic fractions. In growing cells in the presence of 2,4-DNP, the induction of the enzyme paralleled the 2,4-DNP uptake phase (Fig. 2). Nitrophenol reductase activity was not induced in the absence of nitrophenol, and the apparent constitutive activity observed at time zero (Fig. 2) was due to the residual nitrophenol reductase activity of the inoculum, which was composed of cells grown in medium con-

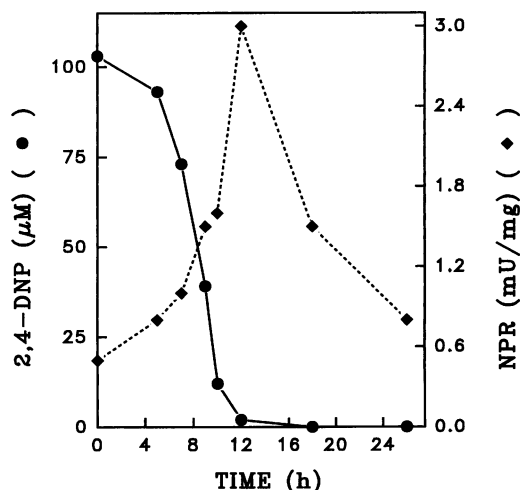


FIG. 2. Induction of nitrophenol reductase (NPR) activity in *Rhodobacter capsulatus* E1F1 cells cultured phototrophically in the presence of 2,4-DNP. Cells growing in medium containing 2,4-DNP were used as the inoculum; these cells were cultured in the presence of light under anaerobic conditions in medium containing 2,4-DNP as described in Materials and Methods. The concentration of 2,4-DNP in the medium and the nitrophenol reductase activities in crude extracts were measured at different times.

taining 2,4-DNP. In the presence of light under microaerobic conditions similar induction of nitrophenol reductase was observed (data not shown).

The specific activity of nitrophenol reductase was always very low in crude extracts obtained from *Rhodobacter capsulatus* E1F1 grown in the presence of 2,4-DNP (up to 3 mU/mg of protein at the log phase of growth). This result suggests that the physiological substrate for the enzyme is some unidentified electron acceptor and not 2,4-DNP. Nitrophenol reductase is probably an inducible reductase which, in the presence of nitrophenols, could function as a detoxifying enzyme.

TABLE 1. Nitrophenol reductase activities in *Rhodobacter capsulatus* E1F1 cells cultured in media containing nitro compounds<sup>a</sup>

Medium containing:	Nitrophenol reductase activity (mU/mg of protein)
2-Nitrophenol.....	0.7
3-Nitrophenol.....	1.3
4-Nitrophenol.....	0.5
2,4-DNP.....	3.0
2,5-Dinitrophenol.....	1.5
3,4-Dinitrophenol.....	0.7
2,4,6-Trinitrophenol.....	2.0
2,4-Dinitrobenzoate.....	<0.5
2,4-DNP + NH <sub>4</sub> Cl.....	0.0
No nitrogen.....	0.0

<sup>a</sup> Cells were cultured in the presence of light under anaerobic conditions with 10 mM acetate as the carbon source and with different nitro compounds. The concentration of nitro compounds used was 0.1 mM; concentration of ammonium chloride used was 6 mM. Cells were harvested in the mid-logarithmic phase of growth, and nitrophenol reductase activities were determined in crude extracts by using 2,4-DNP as the substrate as described in Materials and Methods. No growth was observed in medium containing 2,4-dinitrobenzene, 2,4-dinitrotoluene, or 2,4-dinitroaniline. Except in the experiment with NH<sub>4</sub>Cl, the cells used dissolved N<sub>2</sub> as the nitrogen source for growth.

TABLE 2. Alternative substrates for nitrophenol reductase from *Rhodobacter capsulatus* E1F1<sup>a</sup>

Compound	Nitrophenol reductase activity (mU/mg of protein)
2-Nitrophenol.....	16
3-Nitrophenol.....	0.0
4-Nitrophenol.....	0.0
2,4-DNP.....	42
2,5-Dinitrophenol.....	121
3,4-Dinitrophenol.....	30
2,4,6-Trinitrophenol.....	37

<sup>a</sup> Cells were grown in the presence of light under anaerobic conditions in medium containing 0.1 mM 2,4-DNP and were harvested at the mid-logarithmic phase of growth. The nitrophenol reductase activity of a partially purified fraction (DEAE-Sephacel eluate) was measured by using different substrates as described in Materials and Methods. No activity was found with 2,4-dinitrobenzene, 2,4-dinitrotoluene, or 2,4-dinitroaniline as the substrate of the enzyme.

Nitrophenol reductase activity was found in *Rhodobacter capsulatus* E1F1 cells grown on media containing nitrophenols but not when nitroaromatic derivatives lacking the phenolic group were substituted for nitrophenols in the culture media (Table 1). With the exception of 3- and 4-nitrophenols, the compounds that induced nitrophenol reductase activity were also substrates for the enzyme (Tables 1 and 2). The highest level of activity was found when 2,5-dinitrophenol was the substrate (Table 2). By contrast, other bacterial nitroreductases reduce nonphenolic nitro derivatives (4, 23, 28). Diazotrophic cultures expressed nitrophenol reductase activity if 2,4-DNP was added to the medium, but the enzyme was never detected in cells cultured with ammonium, even in the presence of 2,4-DNP (Table 1). On the other hand, addition of ammonium to growing cells in the presence of 2,4-DNP strongly decreased the nitrophenol reductase activity (data not shown), which is consistent with the previously reported strong inhibition of 2,4-DNP consumption by ammonium in *Rhodobacter capsulatus* E1F1 (1).

Nitrophenol reductase from *Rhodobacter capsulatus* E1F1 used several nitrophenols as substrates (Table 2), although other bacterial nitroreductases exhibited broader substrate specificity (19, 23). Thus, the enzyme from *Rhodobacter capsulatus* E1F1 was not able to reduce 2,4-dinitrobenzene, 2,4-dinitrotoluene, and 2,4-dinitroaniline (Table 2). Like nitrobenzoate reductase from *Nocardia* sp. (4), nitrophenol reductase from *Rhodobacter capsulatus* E1F1 was inhibited by iron chelators and mercurial com-

TABLE 3. Inhibitors of nitrophenol reductase of *Rhodobacter capsulatus* E1F1<sup>a</sup>

Compound	Concn (mM)	% Inhibition
<i>p</i> -HMB <sup>b</sup>	0.5	35
KCN	0.5	10
<i>o</i> -Phenanthroline	0.5	77
Salicylhydroxamate	0.5	40
Neocuproin	1.0	15
CuSO <sub>4</sub>	0.2	95

<sup>a</sup> Partially purified nitrophenol reductase preparations from the gel filtration eluates were assayed as described in Materials and Methods; 100% activity corresponded to 17 mU/mg of protein. NH<sub>4</sub>Cl, KNO<sub>3</sub>, KNO<sub>2</sub>, hydroxylamine, dithioerythritol, and EDTA (sodium salt), which were tested by using concentrations up to 5 mM, had no effect.

<sup>b</sup> *p*-HMB, *p*-hydroxymercuribenzoate.

TABLE 4. Purification of nitrophenol reductase from *Rhodobacter capsulatus* E1F1

Purification step	Vol (ml)	Protein content (mg)	Total activity (mU)	Sp act (mU/mg)	Yield (%)
Crude extract	14	83	158	1.9	100
Ultracentrifugation	14	52	166	3.2	105
Ammonium sulfate	3.8	37	132	3.6	83
Gel filtration	14	4.4	81	18	51
DEAE-Sephacel	9	0.3	16	53	10
Preparative electrophoresis	0.5	0.05	9	180	6

pounds (Table 3), thus suggesting that both iron and sulfhydryl groups are essential for enzyme activity and/or stability. As previously reported for the nitrophenol reductase of *B. fragilis* (13), strong inhibition of the *Rhodobacter capsulatus* enzyme by  $\text{Cu}^{2+}$  was also observed (Table 3).

Nitrophenol reductase from *Rhodobacter capsulatus* E1F1 was purified by a simple method that combined chromatographic steps and preparative electrophoresis (Table 4). Despite the fact that this purification procedure provided a highly purified nitrophenol reductase preparation, a very low yield was obtained because crude extracts contained small amounts of the enzyme. In addition, most of the enzymatic activity was lost during the chromatographic and preparative electrophoresis steps (Table 4), probably because of the extreme lability of the enzyme. Attempts to purify the enzyme by affinity chromatography by using nitrophenyl Sepharose, Blue Sepharose, or Red Sepharose were not successful. A previously described purification procedure for nitrophenol reductase from *B. fragilis* also produced a low yield (13).

Highly purified nitrophenol reductase of *Rhodobacter capsulatus* E1F1 also exhibited NADPH diaphorase activity, which could be stained in slab polyacrylamide gels after nondenaturing electrophoresis by using NBT as the electron acceptor (data not shown).

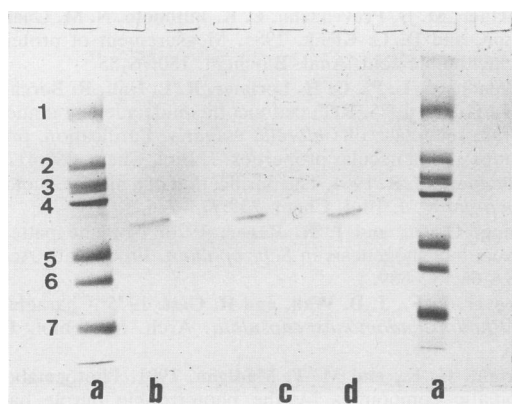


FIG. 3. SDS-PAGE of purified nitrophenol reductase of *Rhodobacter capsulatus* E1F1. Fractions from preparative electrophoresis were subjected to SDS electrophoresis in slab gels. Nitrophenol reductase and protein standards were stained as described in Materials and Methods. Lanes a contained protein standards (standard 1, 66 kDa; standard 2, 45 kDa; standard 3, 36 kDa; standard 4, 29 kDa; standard 5, 24 kDa; standard 6, 20.1 kDa; standard 7, 14.2 kDa); lanes b through d contained consecutive fractions from preparative electrophoresis showing nitrophenol reductase activity.

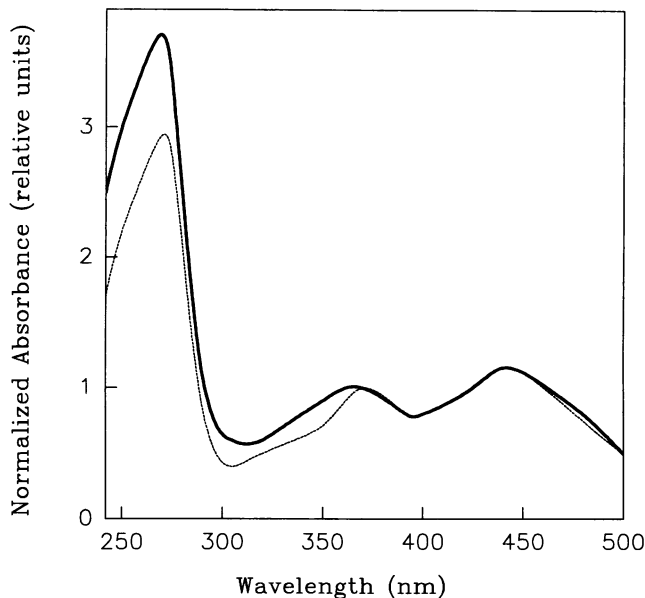


FIG. 4. UV and visible light spectra of a flavin standard and of the chromophore extracted from pure nitrophenol reductase of *Rhodobacter capsulatus* E1F1. Homogeneous nitrophenol reductase was heated at 120°C for 20 min and centrifuged, and the supernatant was injected into an HPLC column as described in Materials and Methods. The HPLC peak was analyzed by a Diode array system which provided the UV and visible light spectra of the sample (solid line) and of an FMN standard (dashed line).

A single 27-kDa protein band was obtained when highly purified nitrophenol reductase from *Rhodobacter capsulatus* was subjected to SDS-PAGE (Fig. 3). A similar size has been reported for the enzyme from *Nocardia* sp. (28), whereas nitrophenol reductase I from *B. fragilis* produced a major 56-kDa band in SDS-PAGE gels (13). Nevertheless, native nitrophenol reductase from *Rhodobacter capsulatus* E1F1 seemed to be a dimer with the following hydrodynamic, molecular, and kinetic parameters: Stokes' radius, 3.061 nm; sedimentation coefficient, 3.196S; frictional quotient, 1.25; molecular mass of native protein, 52 kDa; molecular mass of each subunit, 27 kDa; number of subunit types, 1; number of subunits, 2; optimal temperature, 30°C; optimal pH, 6; apparent  $K_m$  for NADPH, 37  $\mu\text{M}$ ; apparent  $K_m$  for 2,4-DNP, 70  $\mu\text{M}$ . The value for the molecular mass of the nitrophenol reductase of *Rhodobacter capsulatus* agrees with the value reported for nitrophenol reductase I from *B. fragilis* (13). The latter organism possesses other nitroreductase activities with molecular masses ranging from 320 to 680 kDa (13). The optimal pH and optimal temperature of the nitrophenol reductase of *Rhodobacter capsulatus* E1F1 were lower than the corresponding values described for *B. fragilis* nitrophenol reductases (13).

Purification procedures for nitroreductase activities from *Nocardia* sp. strain V (28) and *B. fragilis* (13) have been described previously, but these studies did not examine the prosthetic groups of the enzymes. Reduction of nitroaromatic groups by *Clostridium* and *Nocardia* extracts was stimulated by flavin adenine dinucleotide, but the enzymes responsible for this process were not purified or characterized at the molecular level (4, 23). On the other hand, nitrophenol reductase from *B. fragilis* was activated by FMN (13). Nitrophenol reductase from *Rhodobacter capsu-*

latus E1F1 contains FMN and probably nonheme iron as prosthetic groups. The HPLC chromatograms of samples prepared by heating highly purified nitrophenol reductase were very similar to those obtained by using FMN standards. When the HPLC 9.99-min peak of a sample was analyzed by the Diode array system, the spectrum shown by the solid line in Fig. 4 was obtained. A similar spectrum (Fig. 4, dashed line) was observed when the 9.97-min peak in the HPLC chromatogram of an FMN standard was analyzed.

Nitrophenol reductase from *Rhodobacter capsulatus* E1F1 was inhibited by nonheme iron reagents, such as salicylhydroxamate, and was only weakly inhibited by cyanide, a typical heme ligand (Table 3). In addition, highly purified enzyme did not react with the heme ligand *N,N,N',N'*-tetramethyl benzidine in nondenaturing PAGE gels. In this experiment, catalase was used as a control and was strongly stained (data not shown). These results suggest that nitrophenol reductase from *Rhodobacter capsulatus* E1F1 might contain nonheme iron, in agreement with the role of nonheme proteins as electron carriers in other biological systems exhibiting nitroreductase activity (7, 20).

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