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Purification and Characterization of Ferredoxin-NAD(P)$^+$ Reductase from the Green Sulfur Bacterium *Chlorobium tepidum*

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Abstract

Ferredoxin-NAD(P)$^+$ reductase [EC 1.18.1.3, 1.18.1.2] was isolated from the green sulfur bacterium *Chlorobium tepidum* and purified to homogeneity. The molecular mass of the subunit is 42 kDa, as deduced by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The molecular mass of the native enzyme is approximately 90 kDa, estimated by gel-permeation chromatography, and is thus a homodimer. The enzyme contains one FAD per subunit and has absorption maxima at about 272, 385, and 466 nm. In the presence of ferredoxin and reaction center complex from *C. tepidum*, it efficiently catalyzes photoreduction of both $NADP^+$ and NAD^+. When concentrations of $NADP^+$ exceeded 10 μM, $NADP^+$ photoreduction rates decreased with increased concentration. The inhibition by high concentrations of substrate was not observed with NAD^+. It also reduces 2,6-dichlorophenol-indophenol (DPIP) and molecular oxygen with either NADPH or NADH as efficient electron donors. It showed NADPH diaphorase activity about two times higher than NADH diaphorase activity in DPIP reduction assays at NAD(P)H concentrations less than 0.1 mM. At 0.5 mM NAD(P)H, the two activities were about the same, and at 1 mM, the former activity was slightly lower than the latter.

1. Introduction

In photosynthesis, reaction centers (RCs) convert light energy by photochemical reactions into redox chemical energy, which is further transformed into stable oxidants and reductants such as NAD(P)H and quinols by electron transport chains linked to RCs and by soluble electron transport cofactors.

Green sulfur bacteria grow phototrophically using various reduced inorganic sulfur compounds such as sulfide and thiosulfate, as well as hydrogen, as ultimate electron donors [1]. Their RCs (PS-Cs) belong to an iron-sulfur type or type I RC [see 2,3,4, for reviews], which also includes the photosystem I of oxygenic photosynthetic organisms. These reaction centers reduce ferredoxin (Fd) directly [5]. *Chlorobium tepidum* is a moderately thermophilic bacterium, assimilating CO_2 mainly by the reductive carboxylic acid cycle [6-8]. Reduction reactions occur in several steps in the cycle, and require reduced Fd, reduced FAD, or NAD(P)H for the full operation of the cycle. In oxygenic photosynthesis, NADPH is reduced by PSI with the aid of Fd and Fd-$NADP^+$ reductase (FNR. [EC 1.18.1.3]) [9]. The complete genome of *C. tepidum* has been sequenced at The Institute for Genomic Research (TIGR) and released in a preliminary form. A search of the genomic sequence database revealed a gene for a RubisCO-like protein. Subsequent studies, however, have indicated that the protein is not a *bona fide* RuBisCO [10], a finding which corroborates a general view of the operation of the reductive carboxylic acid cycle in this bacterium [8].

The occurrence of three 2[4Fe-4S] Fds has been predicted (S. Chang and D.A. Bryant, personal communication), all of them expressed under ordinary growth conditions [11]. These Fds were found to support efficient photoreduction of $NADP^+$ by purified RC preparation from *C. tepidum* (PS-C) in the presence of spinach FNR [11].

Weaver et al [12] found that cell extract from *Chlorobium thiosulfatophilum* could reduce NAD^+ and $NADP^+$ under H_2 atmosphere in the presence of Fd and hydrogenase from *Clostridium pasteurianum*. Buchanan and Evans [13] reported that soluble extracts in combination with bacteriochlorophyll-containing particles, both from *Chlorobium thiosulfatophilum,* could reduce $NADP^+$ under illumination in the presence of Fd from *Chromatium*. Kusai and Yamanaka [14] reported partial purification of FNR from *Chlorobium thiosulfatophilum*. The partially purified enzyme was 45 kDa in molecular mass, contained FAD, and showed higher activity toward $NADP^+$/NADPH than toward NAD^+/NADH in several assays at coenzyme concentrations of 1 mM or less. They also found an active fraction with a molecular mass of about 90 kDa. Shioi et al [15] subsequently reported purification of NAD^+ reductase from the green sulfur bacterium *Prosthecochloris aestuarii*. Although their enzyme showed similarities to that of Kusai and Yamanaka [14] in its reactivity with various electron donors/acceptors, and its susceptibility to several inhibitors, it differed from the FNR reported by Kusai and Yamanaka in that it was far more specific for NAD^+/NADH than for $NADP^+$/NADPH. Furthermore, its molecular mass (119 kDa) was considerably greater than the latter.

In the entire genomic sequence of *C. tepidum* released by TIGR, we found no sequences with significant similarities to those coding for enzymes with established FNR activities from various oxygenic photosynthetic organisms, including plants, algae, and cyanobacteria. Although RubisCO activity may be absent and the reductive carboxylic acid cycle is operative in *C. tepidum*, the latter metabolic pathway still requires participation of NAD(P)H in two steps of the cycle [6,7]: reduction of fumarate to malate by malate dehydrogenase [16] and the synthesis of isocitrate from succinate by reductive carboxylation catalyzed by isocitrate dehydrogenase [17].

We have purified a protein with FNR activity from *C. tepidum*, and characterized some of its properties.

2. Material and Methods

2.1. Purification of ferredoxin-NADP reductase

Washed cells of *C. tepidum* (about 75 g, wet weight) were disrupted by passing through a French pressure cell in the presence of protease inhibitors as described by Seo et al. [11]. Extraction and purification of the enzyme were carried out at 4 ^{o}C unless otherwise indicated. The suspension was centrifuged at 20,000 × *g* for 20 min, and the cell debris pellet discarded. The green supernatant was further centrifuged at 160,000 × *g* for 60 min, and the supernatant fractionated with ammonium sulfate between 40 and 80% saturation. Precipitated proteins were collected by centrifugation and either used immediately or stored at –80^{o}C until use. The light-brown colored precipitate was suspended in 20 mM Tris-HCl buffer (pH 7.8) and dialyzed three times against 5 liters of the same buffer for 3 h each time. The dialyzed preparation was cleared by centrifugation at 20,000 × *g* for 20 min, and the supernatant (134 ml) was diluted with two volumes of the same buffer and applied to a DEAE-cellulose column (3.2 cm × 40 cm, DE23 Whatman) equilibrated with the same buffer. After washing the column with about 200 ml of 50 mM Tris-HCl buffer (pH 7.8), the proteins were eluted with a 1200 ml linear gradient of 0 to 800 mM NaCl in 50 mM Tris-HCl buffer (pH 7.8). Fractions (20 ml each) were collected and assayed for NAD(P)H-2,6-dichlorophenol indophenol (DPIP) diaphorase. Active fractions (640 ml) were pooled and concentrated by ultrafiltration to 8 ml (YM-10, Amicon). The concentrate was divided into four 2 ml fractions. Each fraction was applied to a gel-permeation column (Sephacryl S-200 HR 26/60, Pharmacia) equilibrated with 50 mM Tris-HCl buffer (pH 7.8) containing 150 mM NaCl. The proteins were eluted from the column with the same buffer at a flow rate of 30 ml h^{-1}.

Fractions from each of the four column runs were assayed for NAD(P)H diaphorase activity with DPIP as an electron acceptor, and those found rich in NADPH diaphorase were combined. The buffer for these combined fractions (171 ml) was changed to 50 mM MES-NaOH (pH 6.5) by ultrafiltration (YM-10, Amicon). The sample (about 200 ml) was again divided into four 50 ml fractions, each applied to a dye ligand affinity column (1 × 10 cm, Matrex Blue A, Amicon) equilibrated with 50 mM MES-NaOH buffer (pH 6.5). The column was washed with two column volumes of the equilibration medium, and the proteins eluted as 3 ml fractions with a 144 ml linear gradient of 0–1 M KCl in 50 mM MES-NaOH (pH 6.5). The pooled active peak fractions from the four column runs were concentrated as above, their buffer being changed to 50 mM Tris-HCl (pH 7.8) by ultrafiltration (YM-10, Amicon), and were applied to an anion exchange column (Mono Q 10/10, Pharmacia) equilibrated with 50 mM Tris-HCl (pH 7.8) at room temperature. After washing with two column volumes of the same buffer, the proteins were eluted from the column with an 80 ml linear NaCl gradient from 0 mM NaCl in 50 mM Tris-HCl (pH 7.8) to 400 mM NaCl in 50 mM Tris-HCl (pH 6.5), and yielded two major peaks of NADH-diaphorase activity. As the first peak fractions contained high NADPH diaphorase activity, they were concentrated and their buffer was changed to 10 mM MES-NaOH (pH 6.5) by ultrafiltration (YM-10, Amicon). The concentrate was applied to a cation-exchange column (Mono S 5/5, Pharmacia) equilibrated with 10 mM MES-NaOH (pH 6.5) at room temperature. After washing with two column volumes of the buffer, the activity was eluted with a 24 ml linear gradient of 0–200 mM NaCl in MES-NaOH (pH 6.5), yielding single-peak fractions. The combined active peak fractions were brought to 1 M ammonium sulfate, and applied to a hydrophobic column (Phenyl Superose 5/5, Pharmacia) equilibrated with 50 mM Tris-HCl (pH 7.8) containing 1 M ammonium sulfate at room temperature. Proteins were eluted from the column with a 24 ml inverse linear gradient of 1–0 M ammonium

sulfate, yielding a purified enzyme, which was concentrated and their buffer was changed to 50 mM Tris-HCl (pH 7.8) by ultrafiltration (Ultrafree 4, Millipore) and stored at –80 ^{o}C until use.

2.2. Enzyme activities

NAD(P)H oxidase activity, diaphorase activity with DPIP (ε_{600} = 21.8 mM^{-1} cm^{-1}) as an acceptor, and disulfide reductase activity with 5,5'-dithio-bis(2-nitrobenzoate) (DTNB, ε_{412} = 13 mM^{-1} cm^{-1}) were measured as described in [18], [14] and [19], respectively.

Thioredoxin reductase activity was determined as follows: oxidized *Escherichia coli* thioredoxin (T0910, Sigma) was incubated at 220 μM at 25^{o}C with *C. tepidum* FNR or *E. coli* thioredoxin reductase (T7915, Sigma) in 3 ml of a reaction mixture containing 50 mM potassium phosphate (pH 7.0) and 0.1 mM NADPH. After 30 min, the reaction mixtures were quickly cooled on ice, and 50% (w/v) trichloroacetic acid was immediately added to 5% (w/v). After incubation for 5 min, the mixtures were centrifuged at 20,000 × *g* for 20 min, and the precipitates were dissolved in 1 ml of 50 mM potassium phosphate (pH 7.0). DTNB solution was added to each to 0.2 mM, and the absorbance at 412 nm was read after incubation for 5 min at room temperature.

The $NADP^{+}$ photoreduction assay was essentially as described by Seo et al [11] except that both NaCl and Tris-HCl concentrations were 20 mM instead of 50 mM, Fd (FdB, the most active isoform in $NADP^{+}$ reduction [11]) concentration was 4 μM, and bacteriochlorophyll (Bchl) *a* concentration of *C. tepidum* RC complex (PS-C) was 3 instead of 2 μM. FNR and $NAD(P)^{+}$ concentrations were as indicated in figure legends.

2.3. Miscellaneous methods

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis was as described in [20]. Protein bands in the gel (12%) were detected by silver staining (Daiichi Kagaku, Tokyo).

The native molecular mass was deduced by gel-permeation chromatography on Superdex 200 10/30 (Pharmacia) with the buffer containing 50 mM Tris-HCl (pH 7.8) and 150 mM NaCl at a flow rate of 0.5 ml/min with cytochrome *c* (horse heart, 12.4 kDa), carbonic anhydrase (bovine erythrocytes, 29 kDa), albumin (bovine serum, 66 kDa), alcohol dehydrogenase (yeast, 150 kDa), beta-amylase (sweet potato, 200 kDa), blue dextran (2,000 kDa) (MW-GF-200, Sigma) as standards.

For FAD determination, the enzyme was treated with trichloroacetic acid (5%, w/v), and FAD in the neutralized (pH 7.0) supernatant was determined assuming ε_{450} = 11.3 $mM^{-1}cm^{-1}$ as described in [21].

PS-C and FdB were prepared as in [11].

N-terminal amino acid sequence was analyzed by the Edman degradation method with Procise cLC 494 sequencer (Applied Biosystems).

Preliminary genome sequence data of *Chlorobium tepidum* was obtained from The Institute for Genomic Research (TIGR) website at http://www.tigr.org.

3. Results

3.1. Purification of FNR

FNR was purified to apparent homogeneity using the protocol described in Materials and Methods (Table 1). From a DEAE-cellulose column, NADH diaphorase activity eluted as a broad peak with shoulders, while NADPH diaphorase activity eluted in a single smaller peak, slightly behind the main NADH diaphorase peak (data not shown). The NADPH diaphorase peak fractions, which also included a large part of the NADH diaphorase peak, were combined and applied to a Sephacryl S-200 column, from which NADH diaphorase activity eluted in two large peaks with several minor ones. NADPH diaphorase peak activity eluted between the two large NADH peaks (Fig. 1A). NADPH diaphorase peak activity roughly coincided with FNR activity, as determined by reduction of $NADP^+$ by reduced *C. tepidum* Fd (FdB) generated by illuminated PS-C particles. Fractions active in NADPH diaphorase were pooled for further purification. A subsequent Matrex Blue A column effectively removed bulk proteins not adsorbed to the column (data not shown). From a Mono Q column, NADH diaphorase activity eluted in two large peaks, and the NADPH diaphorase peak coincided with the first peak fractions (Fig. 1B). The subsequent Mono S (data not shown) and Phenyl Superose column chromatographies of the pooled NADPH diaphorase fractions purified FNR to apparent homogeneity (Fig. 1C).

The purified FNR migrated as a single band with an apparent molecular mass of 42 kDa, estimated by SDS-PAGE (Fig. 2). The apparent molecular mass of purified native FNR was estimated to be 90 kDa by gel permeation column chromatography (Superdex 200 10/30, Pharmacia) with molecular mass standards as described in Materials and Methods (data not shown). These results indicate that FNR exists as a dimer of two identical polypeptides under the experimental conditions used in this study.

3.2. Basic characters of purified FNR

Purified FNR shows an absorption spectrum typical of a flavoenzyme, with peaks at about 272, 385, and 466 nm (Fig. 3).

In order to determine the species of flavin coenzyme, FNR was treated with trichloroacetic acid, and the fluorescence spectrum of the extracted flavin coenzyme was measured. Based on the ratio of pH-dependent fluorescence intensity, measured at 525 nm (excitation at 366 nm), pH 6.5:pH 2.2 = 0.328, we concluded that the flavin coenzyme of FNR is FAD [22]. From the absorbance of the released FAD [21], the absorption coefficient of the native FNR was calculated to be 10.3 (mM FAD)$^{-1}$ cm^{-1} or 20.6 (mM FNR)$^{-1}$ cm^{-1} at 466 nm. These values were used in calculating the molar concentrations and specific activities of the enzyme in the subsequent studies.

3.3. NAD(P)$^+$ photoreduction activities with C. tepidum PS-C and Fd

In the presence of PS-C and FdB [11] from this bacterium, this enzyme showed Fd-NAD(P)$^+$ photoreduction activity (Fig. 4A, B). This *C. tepidum* enzyme reduces both NAD$^+$ and NADP$^+$. Accordingly, this enzyme is referred to as FNR [EC 1.18.1.3, 1.18.1.2] of the green sulfur bacterium *C. tepidum*. The FNR at 0.05 μM supported NADP$^+$ photoreduction at a rate of 133 μmol NADPH μmol Bchl *a*$^{-1}$ h^{-1} (Fig. 4A). When 5 μM spinach Fd was used instead of FdB from *C. tepidum*, the photoreduction rate was 43 μmol NADPH Bchl *a*$^{-1}$ h^{-1} (data not shown). At coenzyme concentrations of less than 10 μM, the rates of NAD$^+$ photoreduction were slightly higher than those of NADP$^+$ photoreduction. When concentrations of NADP$^+$ exceeded 10 μM, NADP$^+$ photoreduction rates decreased with increased concentration. The pronounced inhibition by high concentrations of substrate was not observed with NAD$^+$ (Fig. 4B).

3.4. Diaphorase activities

Some flavoenzymes have the ability to reduce artificial electron acceptors such as DPIP with NAD(P)H as electron donors (diaphorase activity). The purified *C. tepidum* FNR showed NADPH diaphorase activity about two times higher than NADH diaphorase activity in DPIP reduction assays at NAD(P)H concentrations less than 0.1 mM (Fig. 5). At 0.5 mM NAD(P)H, the two activities were about the same, and at 1 mM, the former activity was slightly lower than the latter. The *C. tepidum* FNR also showed high rates of NAD(P)H oxidase activity (Table 2). Although this FNR reduces DTNB at a considerable rate, reduction of thioredoxin from *E.coli* with NADPH as the reductant was not detectable (<0.1 μmol thioredoxin min^{-1} μmol FNR^{-1}) (Table 2).

3.5. N-terminal amino acid sequence and similarity with other protein sequences

The 25 N-terminal amino acid residues of purified *C. tepidum* FNR were sequenced, and this sequence is compared to those of other similar proteins in Fig. 6.

4. Discussion

Several of the properties of purified FNR from *C. tepidum* are similar to those of the same enzyme purified from *C. thiosulfatophilum* by Kusai and Yamanaka [14]. These properties include photoreduction of both $NADP^+$ and NAD^+ by reduced Fd with very high affinities (Fig. 4B), oxidation of both NADPH and NADH with DPIP as the acceptor with high affinities (Fig. 5), and having FAD as the flavin cofactor. The two preparations differ, however, in molecular mass. We found that *C. tepidum* FNR is a dimeric protein of 90 kDa, whereas *C. thiosulfatophilum* FNR eluted from a Sephadex G-100 column in two peaks with apparent molecular masses of 90 (Peak I) and 45 kDa (Peak II). Kusai and Yamanaka [14] published enzymatic activities of the Peak II fraction. They suggested that though Peaks I and II differed in molecular mass, they contained the same enzyme because both peak fractions showed qualitatively the same enzymatic activity in several reactions with $NAD(P)^+$/NAD(P)H as substrates. Quantitative comparisons of the activities between the two fractions were not described, however. To determine if a low molecular form of FNR appears during the course of purification, we monitored the molecular mass of *C. tepidum* FNR by gel-permeation chromatography (Superdex-200 10/30, Pharmacia) at several purification steps subsequent to Sephacryl S-200 chromatography. We found that *C. tepidum* FNR always eluted as a single peak of about 90 kDa under the conditions tested.

Shioi et al. [15] purified an FAD-containing flavoprotein from the green sulfur bacterium *Prosthecochloris aestuarii*, which was 119 kDa in molecular mass. They showed that it considerably accelerated the reduction of NAD^+ (NAD^+ reductase) in the presence of illuminated thylakoids and Fd, both from spinach. Their assays of enzymatic activity were compromised, however, by the use of spinach thylakoids only partially depleted in FNR. Such preparations are still active in $NADP^+$ reduction without the addition of FNR. As a

result, the effects of enzyme addition on $NADP^+$ photoreduction were less than the effects on NAD^+ reduction. Their NAD^+ reductase catalyzed the reduction of NAD^+ (0.2 mM), using reduced benzyl viologen as an electron donor, about 5 times faster than the reduction of $NADP^+$. The diaphorase activity of the enzyme was far more specific for NADH than for NADPH. It is evident that our *C. tepidum* FNR significantly differs from *P. aestuarii* NAD^+ reductase in molecular mass and in relative affinities for NAD^+/NADH and $NADP^+$/NADPH in several reactions. We thus conclude that the two proteins are different. As shown in Fig. 1A, *C. tepidum* contains at least three NAD(P)H diaphorase-active enzymes. As diaphorase assay is not very specific for FNR, we measured $NADP^+$ photoreduction activity in the crude fractions obtain from the Sephacryl S-200 column. We were unsuccessful in reliably measuring NAD^+ photoreduction activity in these fractions, however, probably because photoreduced NADH was quickly reoxidized by NADH-oxidizing activities copurified in the same fractions. Since extracts from *C. tepidum* cells contained NADH diaphorase activity much higher than those of NADPH diaphorase activity under our assay conditions (Fig. 1A), we cannot exclude the possibility that *C. tepidum* cells contain an enzyme that preferentially supports reduction of NAD^+ with reduced Fd as an electron donor as reported by Shioi et al [15] but we did not study it further.

C. tepidum FNR thus differs from the FNR of oxygen-evolving photosynthetic organisms both in being a dimer, rather than a monomer, and in having significantly larger monomer molecular mass. *C. tepidum* FNR has high NAD(P)H oxidase activity in the absence of Fd, amounting to about 5-10% of DPIP-NAD(P)H diaphorase activity measured at 0.15 mM NAD(P)H (Table 2, Fig. 5). On the other hand, the NADPH oxidase activity of spinach FNR was about 0.3% of the DPIP-NADPH diaphorase activity at 0.1 mM NADPH [23]. The activity ratio of NADPH oxidase to diaphorase is thus much higher with *C.*

tepidum FNR than with spinach FNR, indicating that the latter has evolved to decrease its reactivity toward oxygen significantly. Both $NADP^+$ reductase activity (Fig. 4) and NADPH diaphorase activity (Fig. 5) are inhibited at high concentrations of $NADP^+$ or NADPH. The reason for this is not certain, but similar observations were made on spinach FNR. Forti and Sturani [24] found that NADPH inhibit NADPH-cyt *c* reductase activity competitively with Fd. Batie and Kamin [25] reported that, in the ternary complex formation ($Fd{\cdot}FNR{\cdot}NADP^+$), $Kd_{(Fd)}$ increased with $NADP^+$ concentration and $Kd_{(NADP^+)}$ also increased on addition of excess Fd.

We determined the sequence of the first 25 N-terminal amino acid residues of *C. tepidum* FNR (Fig. 6). The whole genome of *C. tepidum* has been sequenced at TIGR and is available to the public in a preliminary form. We performed a BLAST search of the *C. tepidum* genomic database using the N-terminal amino acid sequence of purified FNR from *C. tepidum* (Fig. 6). We found a single ORF encoding a thioredoxin reductase-like protein (TRLP) with 100% identity at the N-terminal 25 amino acids of our purified FNR. The TRLP is composed of 360 amino acid residues and has a deduced molecular mass of 39.2 kDa in the absence of bound FAD. We tentatively conclude that our FNR is TRLP.

We also performed a BLAST search with the deduced amino acid sequence of *C. tepidum* TRLP, on the GenomeNet server (Kyoto University) using the EMBL database (GP), National Biomedical Research Foundation database (PIR), Protein Research Foundation databse (PRF, Osaka), and found five putative thioredoxin reductase homologs with E values $<10^{-54}$. These were *Bacillus halodurans* thioredoxin reductase (AP001518_239,GP), *Bacillus subtilis ycgT* (G69759, PIR) and *yumC* (B70015, PIR), *Rickettsia prowazekii* thioredoxin reductase (*trxB2*) RP514 (D71655, PIR) and *Sulfolobus solfataricus* NAD(P)H oxidase: isotype NOX38 (2606382A, PRF) (Fig. 6A). *C. tepidum*

TRLP also shows significant similarity to more than 50 known or putative bacterial thioredoxin reductases (10^{-22}<E <10^{-7}) including one of *E. coli* (E = 2×10^{-8}), as well as to alkyl hydroperoxidase reductase subunit F and the glutamate synthase small subunit. These known or putative thioredoxin reductases exist as homo-dimers [26] and contain the two-cysteine motif found in thioredoxin reductases (see below), absent in the top-scored 6 TRLPs including one of *C. tepidum* (Fig. 6A). We found that although *C. tepidum* FNR can reduce DTNB at appreciable rates in the absence of thioredoxin, it cannot reduce *E. coli* thioredoxin (Table 2).

The amino acid sequence identity of the deduced *C. tepidum* TRLP with angiosperm FNRs was low (E>10^{-3})(see also Fig. 7). *C. tepidum* TRLP protein also differs from *Escherichia coli* FNR [27] and *Azotobacter vinelandii* FNRs (*fpr* products, [28]) which are monomeric proteins having FAD.

Further analysis of the deduced amino acid sequence with the program InterPro [29], shows that *C. tepidum* TRLP shares several motifs with the FAD-dependent nicotinamide nucleotide reductase family [26]. Although bacterial thioredoxin reductase and alkyl hydroperoxidase reductase contain conserved disulfide-reduction activity in two-cysteine motifs ACA(V/T/I)CDG [30] and YCPHCDG [31], respectively, these two-cysteine motifs are absent from *C. tepidum* TRLP. These two-cysteine motifs are also absent from the five top-scoring putative thioredoxin reductases (Fig. 6A). A search of the database at TIGR revealed that *C. tepidum* has a gene which encodes an additional protein with very high similarity to *E. coli* thioredoxin reductase [32] (E = 8×10^{-85}) and with a conserved two-cysteine motif ACATCDG found in *bona fide* thioredoxin reductases (Fig. 6B).

A phylogenetic tree of FNR, thoredoxin reductase and related proteins has been developed (Fig. 7) in which *C. tepidum* FNR is more closely related to *bona fide* thioredoxin reductases than to monomeric FNRs of oxgenic photosynthetic organisms. We

thus conclude that *C. tepidum* TRLP is not a thioredoxin reductase, but rather a previously undescribed type of FNR which may have diverged from the thioredoxin reductase family in the course of evolution.

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Figure Legends

Fig. 1. Fractionation of FNR by Sephacryl S-200 (A), Mono Q 10/10 (B) and Phenyl Superose (C) chromatographies. Continuous line with or without closed circle (●) in (A)~(C), A_{280}; dotted lines with open triangle (Δ) in (A) and (B), NADPH diaphorase activity ([NADPH] = 0.1 mM, [DPIP] = 0.1 mM); dotted lines with open square (□) in (A) and (B), NADH-diaphorase activity ([NADH] = 0.1 mM, [DPIP] = 0.1 mM); dotted line with cross (+) in (A), $NADP^+$ photoreduction activity with PS-C and *C. tepidum* FdB in the presence of 400 μl sample as indicated ([$NADP^+$] = 0.1 mM). Experimental conditions are as described in Materials and Methods. Fraction volume; 2 ml (A) and 2.2 ml (B). FNR active fractions pooled for the next purification step are indicated by bars.

Fig. 2. SDS-PAGE analysis of purified FNR from *C. tepidum*. Lane 1, 66 ng of purified FNR. Protein amount was determine by Protein Assay reagent kit (BioRad) using BSA as standard; lane 2, molecular mass standards (Silver stain SDS-PAGE standard, BioRad).

Fig. 3. The ultra violet–visible absorption spectrum of purified FNR from *C. tepidum*. Sample was dissolved in 50 mM Tris-HCl Buffer (pH 7.8) containing 150 mM NaCl. An enlargement of the spectrum in the blue region is shown in the inset.

Fig. 4. (A) Relationship between the rates of $NADP^+$ photoreduction and FNR concentration. In addition to those described in Materials and Methods, the reaction mixture contained indicated concentration of FNR and 1 μM (○), 10 μM (■), 20 μM (▲), 50 μM (x), 100 μM (✳), or 200 μM (●) $NADP^+$. The optical cells containing reaction mixtures were illuminated under strictly anaerobic conditions at 23°C with yellow light (Y-

48, Toshiba) at 1300 μmol $m^{-2}s^{-1}$ for 1 min during which time the absorbance changes at 340 nm were monitored. (B) Relationship between the rates of $NAD(P)^+$ photoreduction and $NAD(P)^+$ concentration. The mixture contained 0.025 μM FNR and the indicated concentration of NAD^+ (●) or $NADP^+$ (○).

Fig. 5. Dependence of DPIP-diaphorase activity on NADH (●) and NADPH (○) concentration. The reaction mixture contained 0.01 μM *C. tepidum* FNR, 0.05 mM DPIP, 50 mM potassium phosphate (pH 7.0) and the indicated concentration of NAD(P)H. The reaction was initiated with the addition of FNR and the absorbance change at 600 nm was measured.

Fig. 6. Multiple alignment of deduced amino acid sequences of (A): TRLP from *C. tepidum* (TIGR, unpublished), putative thioredoxin reductase (*trxB2*) RP514 of *Rickettsia prowazekii* (D71655, PIR), *yumC* gene of *Bacillus subtilis* (B70015, PIR), *Bacillus halodurans* thioredoxin reductase (AP001518_239,GP), *ycgT* gene of *Bacillus subtilis* (G69759, PIR) and NADH oxidase of *Sulfolobus solfataricus* (2606382A, PRF), and (B): putative thioredoxin reductase from *C. tepidum* (TIGR, unpublished) and thioredoxin reductase from *E. coli* [31]. Alignment data were obtained using CLUSTALW 1.8. The initial 25 N-terminal amino acid residues of purified *C. tepidum* FNR determined by protein sequencing are underlined. The amino acid residues conserved or conservatively substituted among all six sequences in (A) are boxed. Asterisks indicate identical amino acid residues and dots indicate residues that are identical in at least six or seven among all eight listed sequences. The conserved two-cysteine motif of disulfide reductases is indicated in boldface in (B). For details, see text.

Fig. 7. Phylogenetic tree of amino acid sequences of ferredoxin-$NADP^+$ reductases (FNR), NADPH-thioredoxin reductases and its homologs. The tree was constructed by the neighbor-joining method on CLUSTALW 1.8. The lengths of lines indicate the relative distances between nodes. TRLP: NADPH-thoredoxin reductase-like protein, TRR: *bona fide* or putative NADPH-thioredoxin reductase. *Arabidopsis, Arabidopsis thaliana*; *A. vinelandii*, *Azotobacter vinelandii*; *B. halodurans*, *Bacillus halodurans*; *B. subtilis*, *Bacillus subtilis*; *C. reinhardtii*, *Chlamydomonas reinhardtii*; *C. tepidum*, *Chlorobium tepidum*; *E. coli, Esherichia coli*; *R. prowazekii*, *Rickettsia prowazekii*; *S. cerevisiae*, *Saccharomyces cerevisiae*; spinach, *Spinacia oleracea*; *S. solfataricus*, *Sulforobus solfataricus*. For detail, see text and the legend to Fig. 6.

Table 1 Purification of FNR from *C. tepidum*

Purification Step	Protein amount (mg)	Activity[a] (U)	Purification factor	Recovery (%)
Supernatant	7810	81.3	1	100
40% ~ 80% SAS[b]	7270	64.8	0.89	79.6
DEAE	3410	65.2	1.9	80.2
Sephadex S-200	971	46.0	4.7	56.6
Matrex Blue A	156	45.4	29.1	55.9
Mono Q	33.8	42.4	125	52.2
Mono S	1.26	11.2	890	13.8

[a] Activity was checked by diaphorase assay with 0.1 mM NADPH and 0.1 mM DPIP.

[b] saturated ammonium sulfate

Table 2 Enzymatic activities of *C. tepidum* FNR

Oxidase assay	
NADPH	1.7×10^2 μmol NADPH min^{-1} μmol FNR^{-1} (at 0.15 mM NADPH)
NADH	0.9×10^2 μmol NADH min^{-1} μmol FNR^{-1} (at 0.15 mM NADH)
DTNB reductase	
NADPH	2.8×10 μmol DTNB min^{-1} μmol FNR^{-1} (at 0.5 mM NADPH)
NADH	9.6×10 μmol DTNB min^{-1} μmol FNR^{-1} (at 0.5 mM NADH)
Thioredoxin reductase	
NADPH	not detectable (< 0.1 μmol thioredoxin min^{-1} μmol FNR^{-1})

Figure 1A

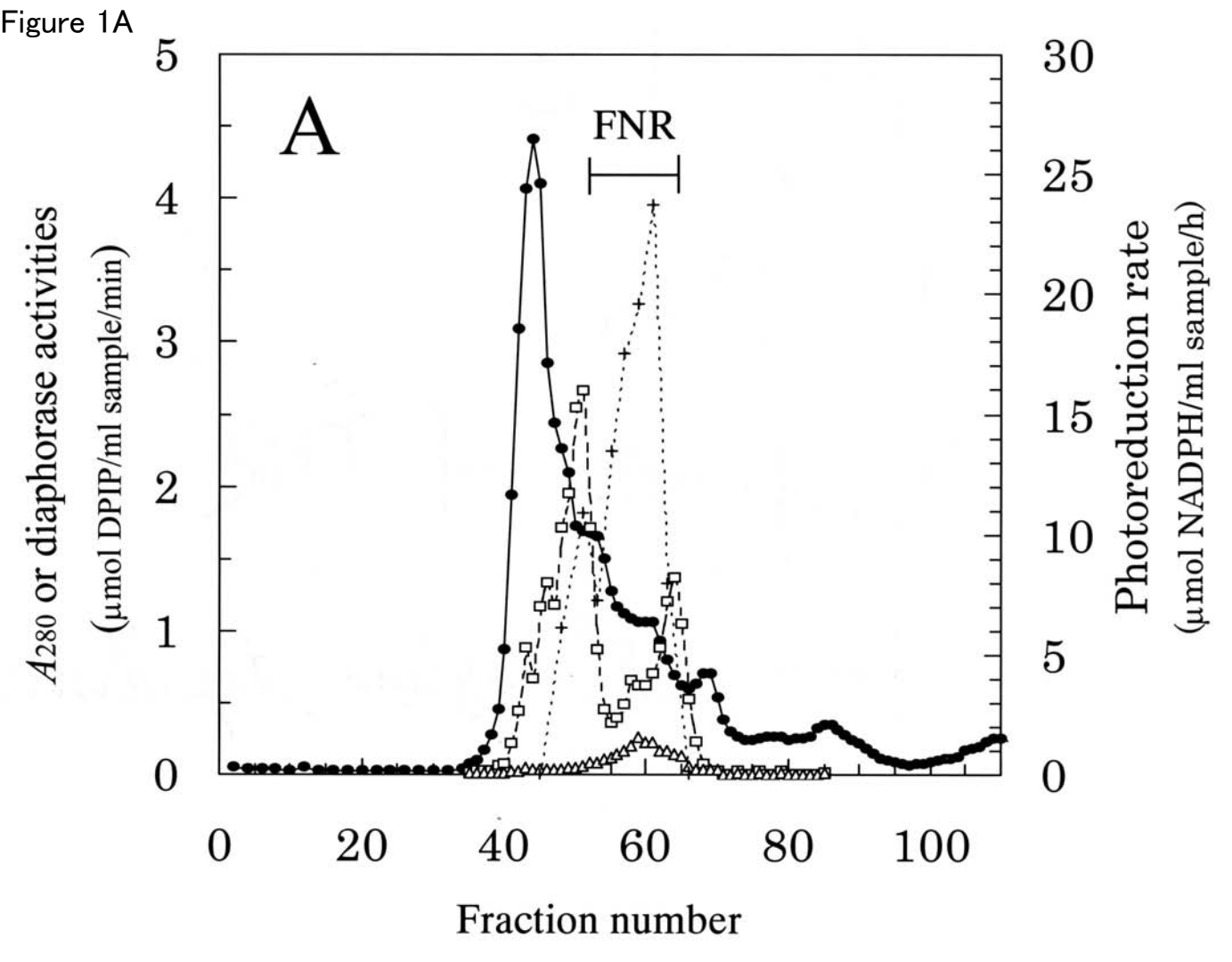

A
FNR
A_{280} or diaphorase activities
(μmol DPIP/ml sample/min)
Photoreduction rate
(μmol NADPH/ml sample/h)
Fraction number
0
1
2
3
4
5
0
5
10
15
20
25
30
0
20
40
60
80
100

Figure 1B

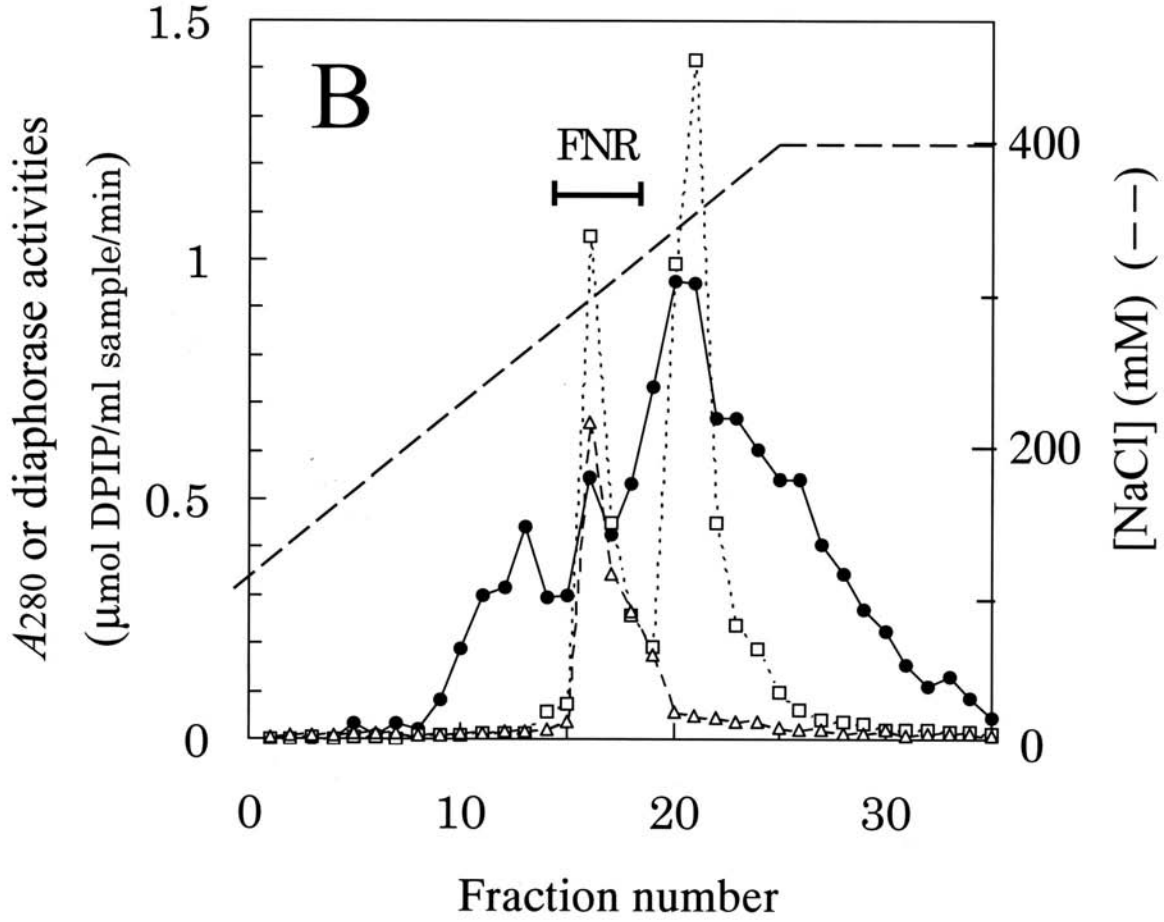

B
FNR
1.5
1
0.5
0
400
200
0
0
10
20
30
A280 or diaphorase activities
(μmol DPIP/ml sample/min)
[NaCl] (mM) (– –)
Fraction number

Figure 1C

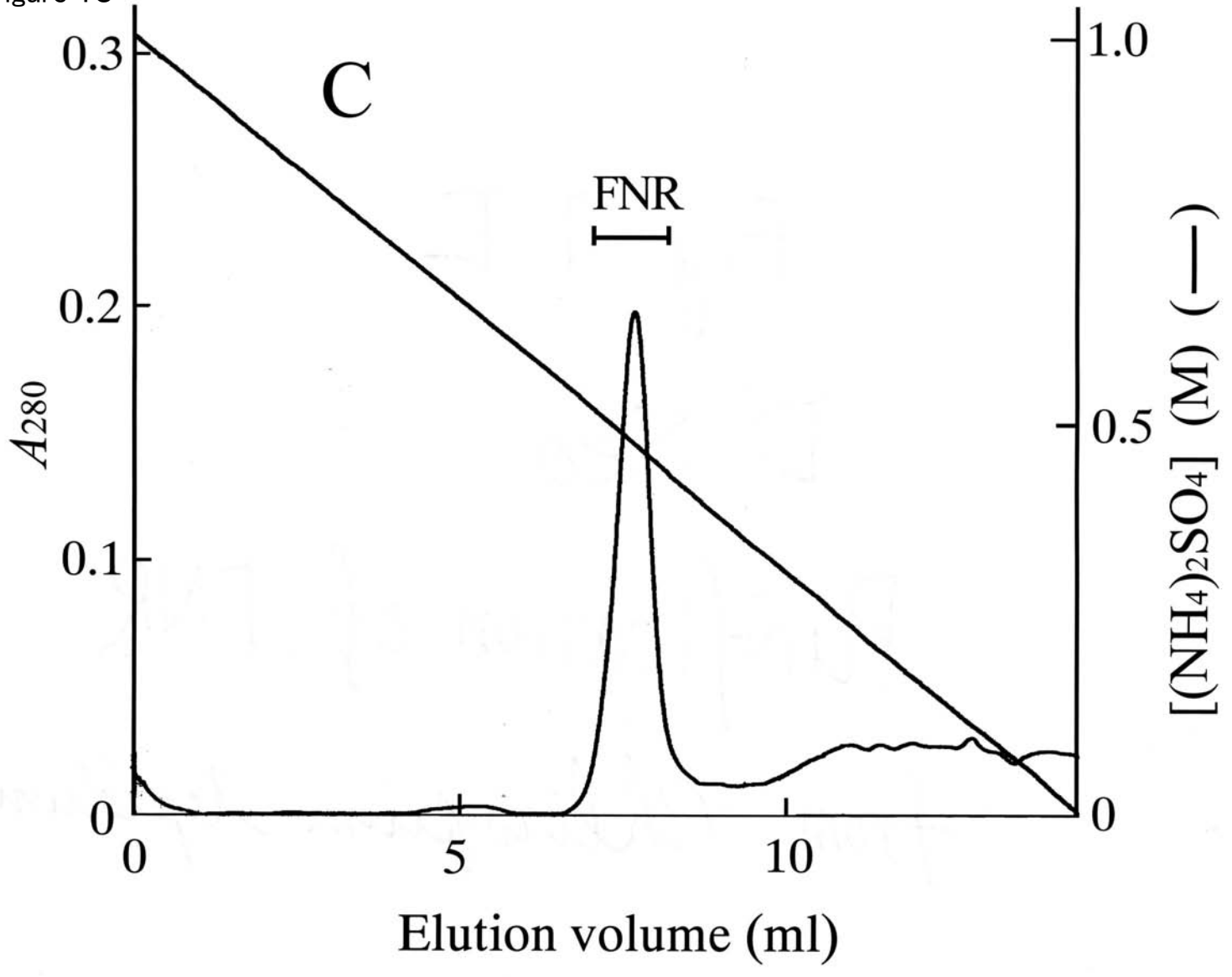


Figure 2

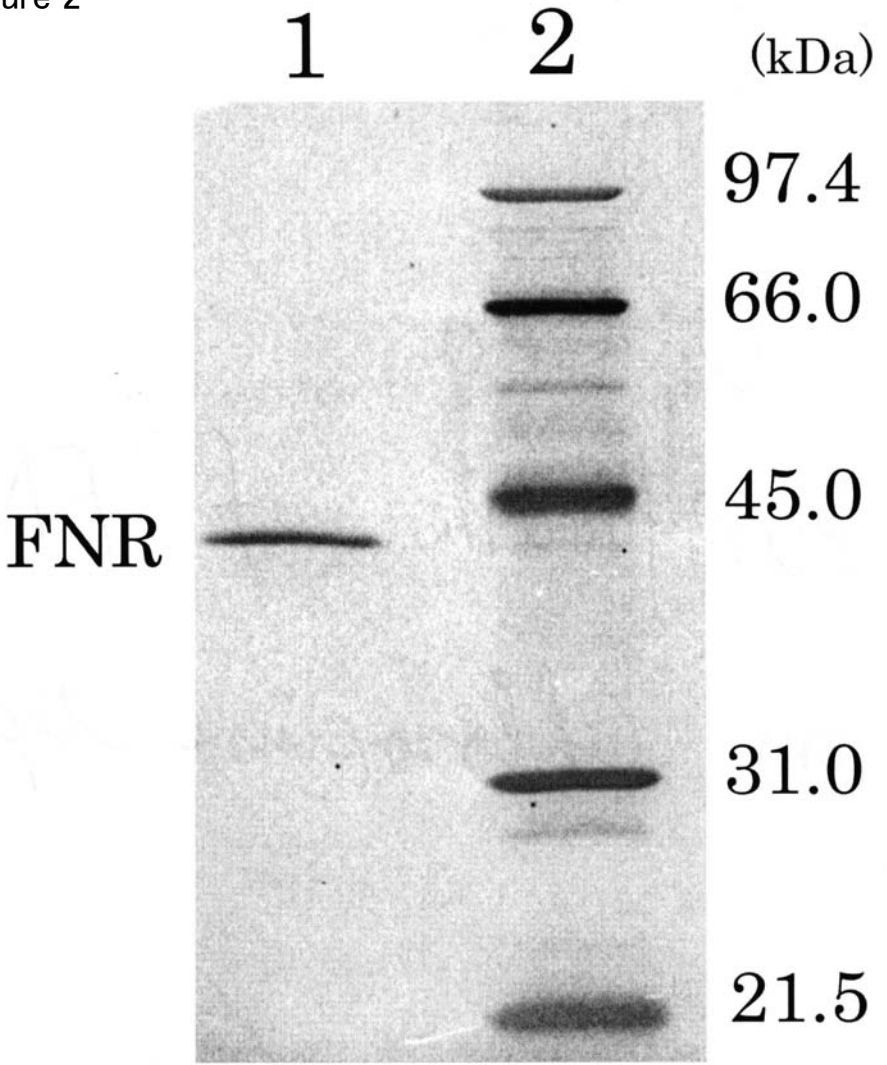


Figure 3

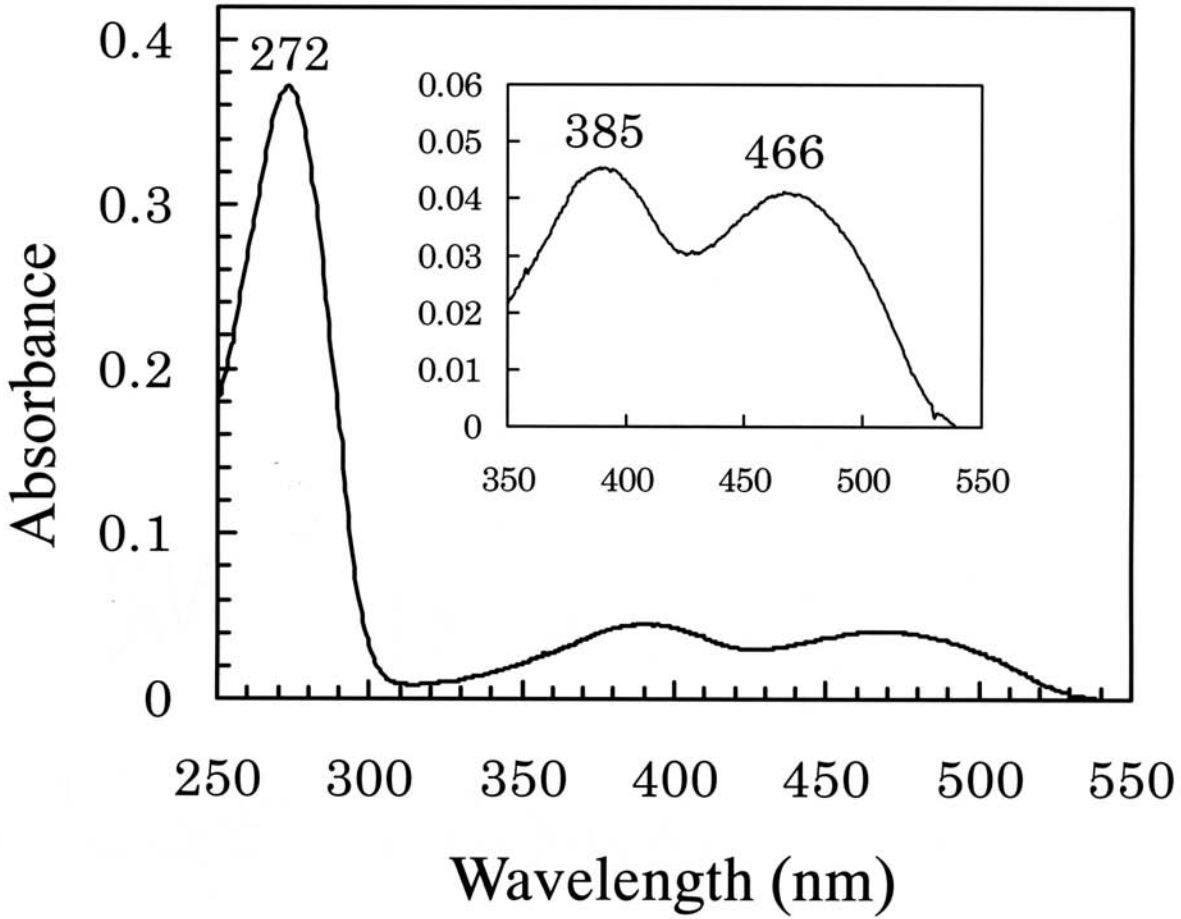
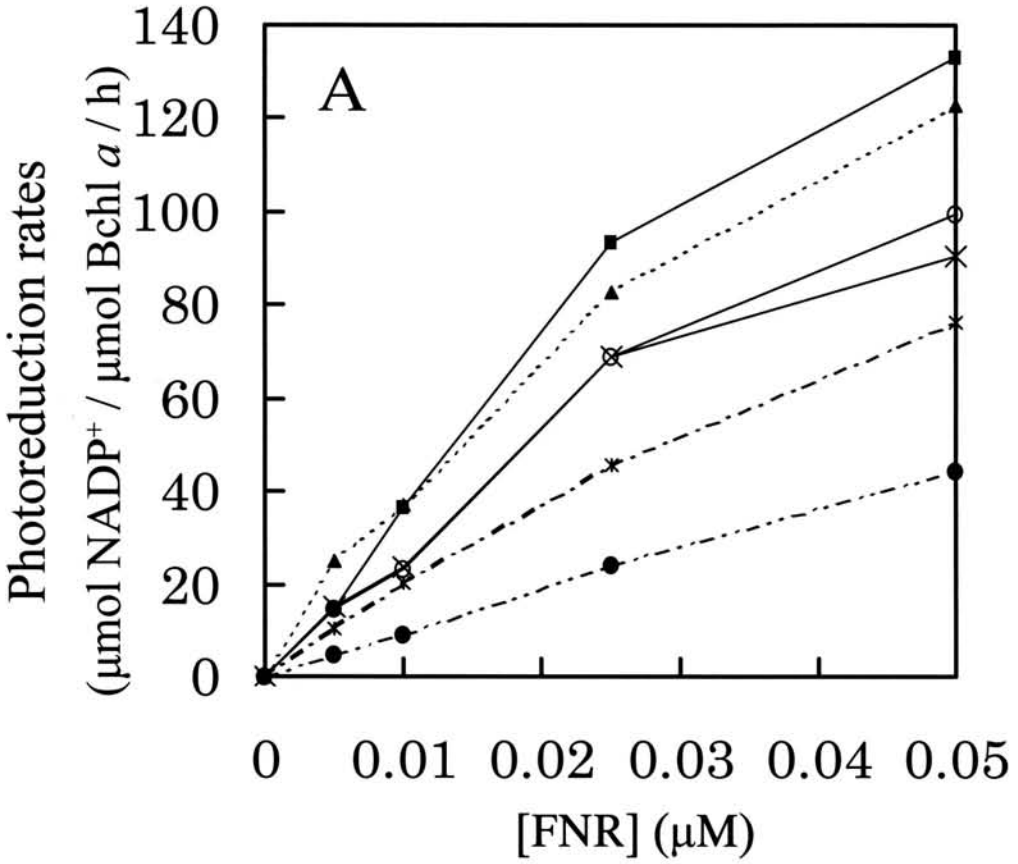

272
385
466
Absorbance
Wavelength (nm)
0.4
0.3
0.2
0.1
0
250
300
350
400
450
500
550
0.06
0.05
0.04
0.03
0.02
0.01
0
350
400
450
500
550

Figure 4


A
Photoreduction rates
(μmol NADP+ / μmol Bchl a / h)
140
120
100
80
60
40
20
0
0
0.01
0.02
0.03
0.04
0.05
[FNR] (μM)

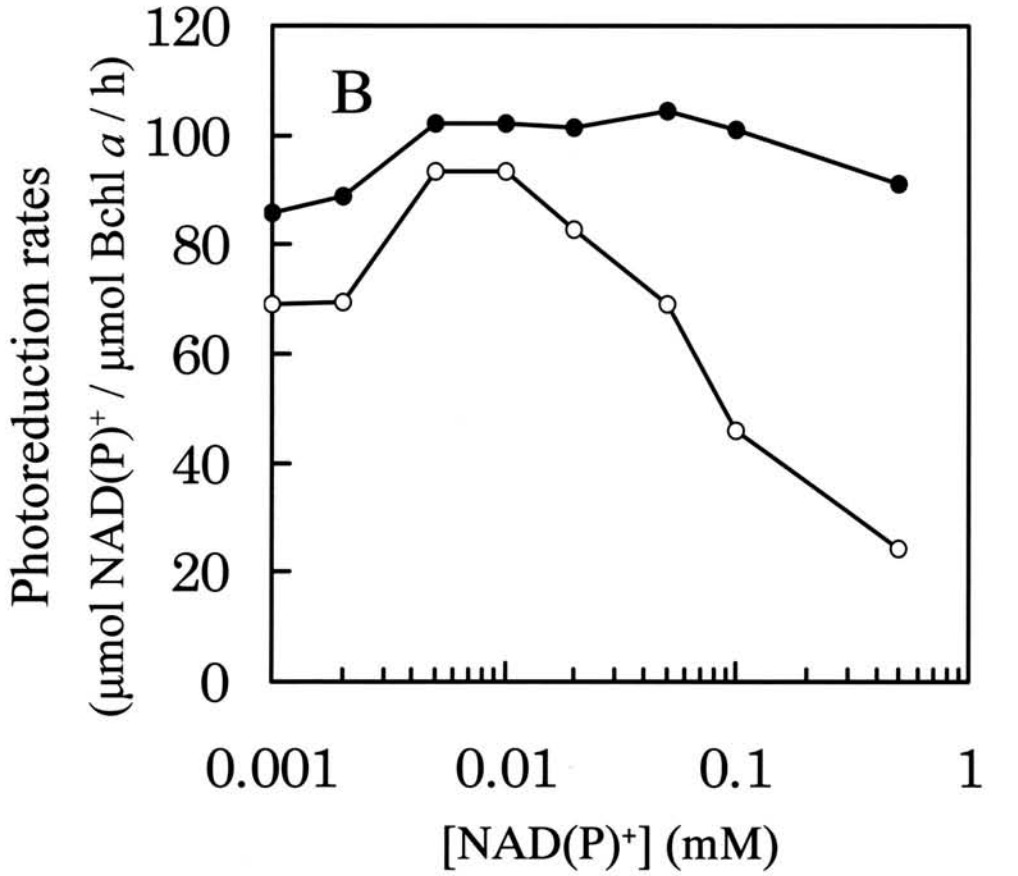

B
Photoreduction rates
(μmol NAD(P)+ / μmol Bchl a / h)
120
100
80
60
40
20
0
0.001
0.01
0.1
1
[NAD(P)+] (mM)

Figure 5

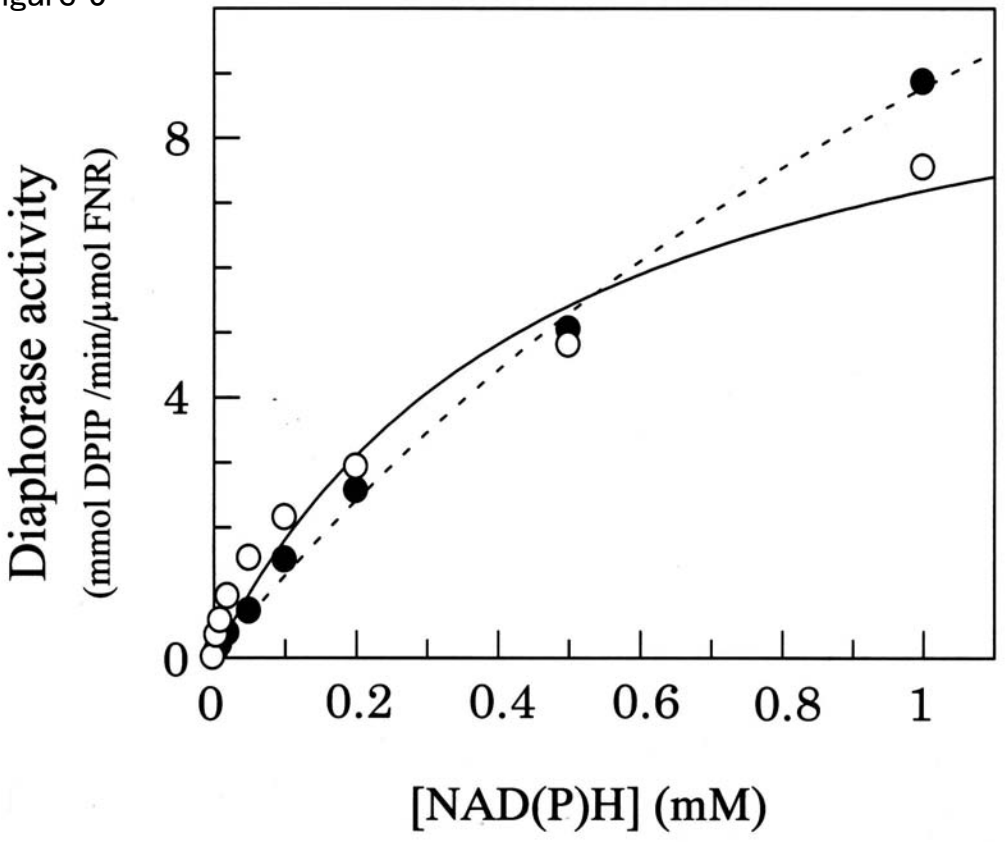

Diaphorase activity
(mmol DPIP /min/μmol FNR)
8
4
0
0
0.2
0.4
0.6
0.8
1
[NAD(P)H] (mM)

Figure 6

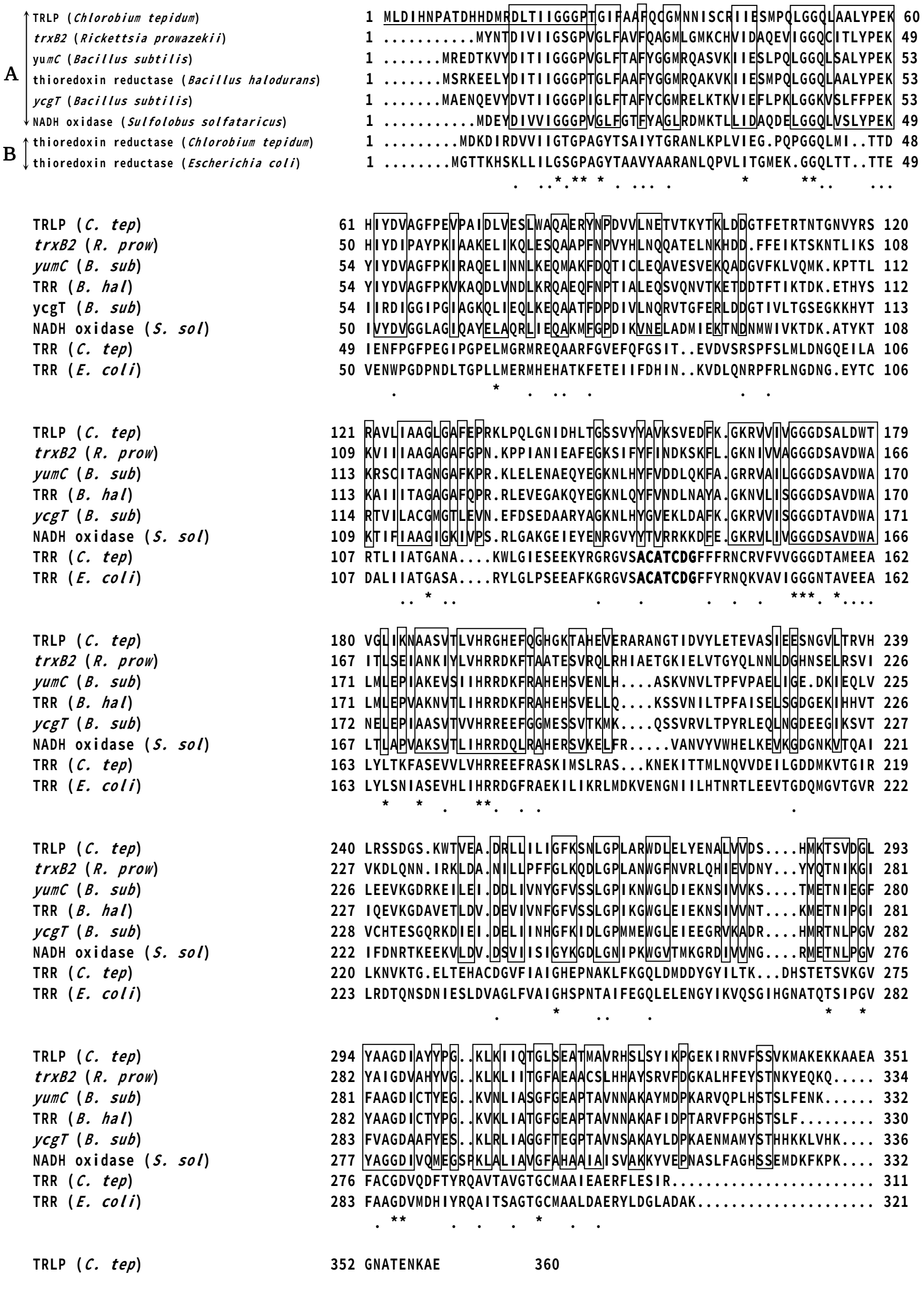
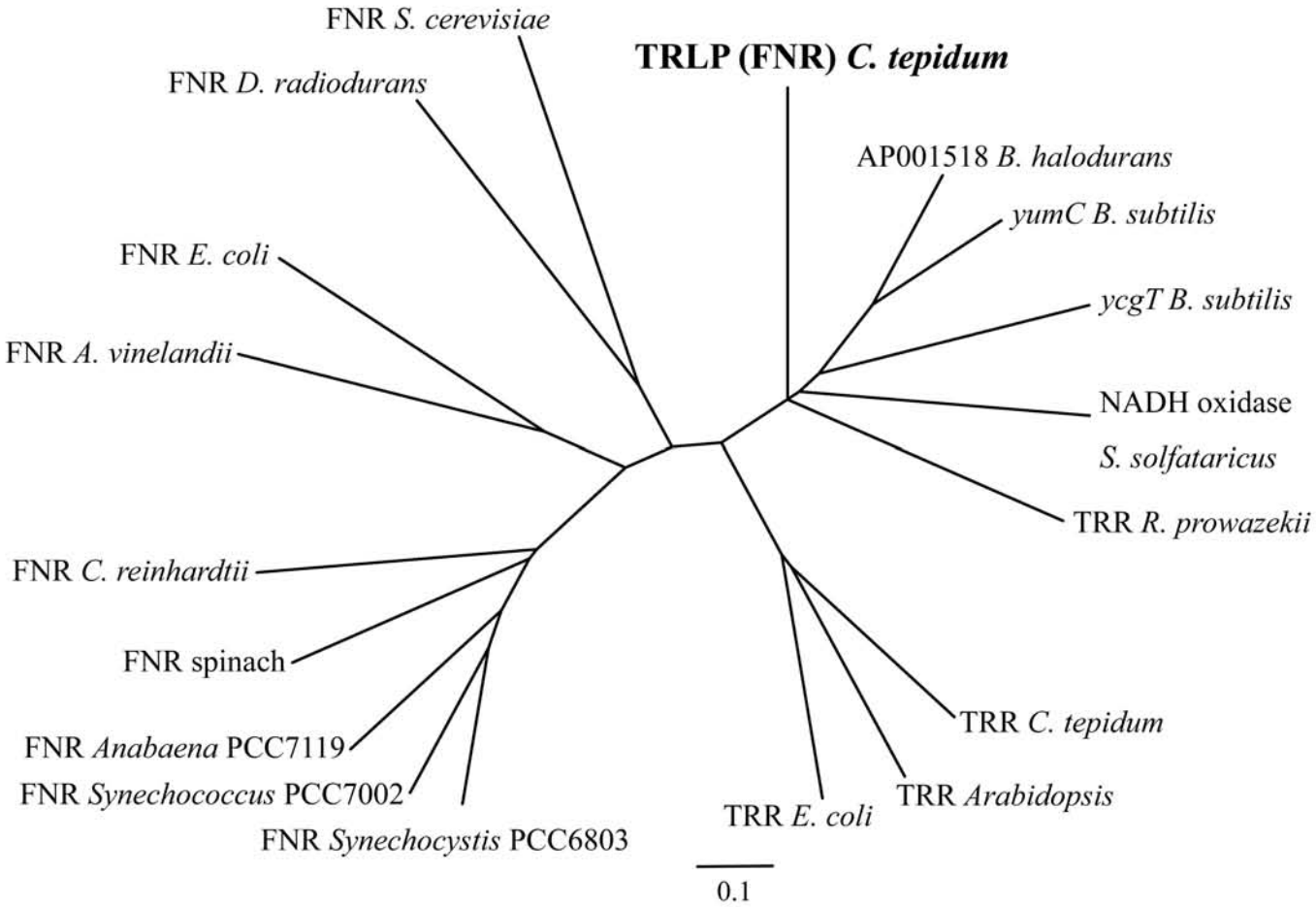

A
B
TRLP (Chlorobium tepidum)
trxB2 (Rickettsia prowazekii)
yumC (Bacillus subtilis)
thioredoxin reductase (Bacillus halodurans)
ycgT (Bacillus subtilis)
NADH oxidase (Sulfolobus solfataricus)
thioredoxin reductase (Chlorobium tepidum)
thioredoxin reductase (Escherichia coli)
TRLP (C. tep)
trxB2 (R. prow)
yumC (B. sub)
TRR (B. hal)
ycgT (B. sub)
NADH oxidase (S. sol)
TRR (C. tep)
TRR (E. coli)
ACATCDG

Figure 7


FNR S. cerevisiae
TRLP (FNR) C. tepidum
FNR D. radiodurans
AP001518 B. halodurans
yumC B. subtilis
FNR E. coli
ycgT B. subtilis
FNR A. vinelandii
NADH oxidase
S. solfataricus
TRR R. prowazekii
FNR C. reinhardtii
FNR spinach
TRR C. tepidum
FNR Anabaena PCC7119
FNR Synechococcus PCC7002
TRR Arabidopsis
TRR E. coli
FNR Synechocystis PCC6803
0.1