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Purification of ferredoxins and their reaction with purified reaction center complex from the green sulfur bacterium *Chlorobium tepidum*

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Abstract

Four ferredoxin (Fd) fractions, namely, FdA–D were purified from the green sulfur bacterium *Chlorobium tepidum*. Their absorption spectra are typical of 2[4Fe–4S] cluster type Fds with peaks at about 385 and 280 nm and a shoulder at about 305 nm. The A_{385}/A_{280} ratios of the purified Fds were 0.76–0.80. Analysis of the N-terminal amino acid sequences of these Fds (15–25 residues) revealed that those of FdA and FdB completely agree with those deduced from the genes, *fdx3* and *fdx2*, respectively, found in this bacterium (Chung and Bryant, personal communication). The N-terminal amino acid sequences of FdC and FdD (15 residues) were identical, and agree with that deduced from the gene *fdx1* (Chung and Bryant, personal communication). The A_{385} values of these Fds were unchanged when they were stored for a month at −80°C under aerobic conditions and decreased by 10–15% when they were stored for 6 days at 4°C under aerobic conditions, indicating that they are not extremely unstable. In the presence of Fd-NADP$^+$ reductase from spinach, and a purified reaction center (RC) preparation from *C. tepidum* composed of five kinds of polypeptides, these Fds supported the photoreduction of NADP$^+$ at room temperature with the following K_m and V_{max} (in μmol NADP$^+$ μmol BChl a^{-1} h^{-1}): FdA, 2.0 μM and 258; FdB, 0.49 μM and 304; FdC, 1.13 μM and 226; FdD, 0.5 μM and 242; spinach Fd, 0.54 μM and 183. The V_{max} value of FdB was more than twice that previously reported for purified RC preparations from green sulfur bacteria.

Keywords: Ferredoxin; Green sulfur bacterium; NADP$^+$ reduction; Photosynthesis; Reaction center

1. Introduction

Green sulfur bacteria are phototrophs which use various inorganic sulfur compounds as electron donors and require strict anaerobic conditions for growth. In the photosynthetic reaction center (RC) of green sulfur bacteria (PS-C), the terminal electron acceptors are iron–sulfur clusters. These PS-Cs belong to an iron–sulfur type, like the RCs of heliobacteria, and the photosystem I (PSI) of oxygenic photosynthetic organisms (see [1,2] for reviews). On

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the other hand, RCs of purple bacteria and green filamentous sliding bacteria contain no iron–sulfur clusters and belong to a pheophytin–quinone type like those of the photosystem II (PSII) of oxygenic photosynthetic organisms. Illuminated PS-C can directly reduce ferredoxin (Fd) [3], but the RC of purple bacteria cannot. The latter organisms reduce Fd via an energy-dependent reversal of electron transfer [4]. The primary donor of these RCs is a special pair of chlorophyll or bacteriochlorophyll (BChl) molecules, which is held at the interface between two RC core polypeptides. The core polypeptides of purple bacterial RC, PSI and PSII are heterodimeric, while those of PS-C [5] and heliobacterial RCs are homodimeric [6]. In purple bacterial RCs, the difference in amino acid sequence between similar RC core polypeptides explains why only one out of two apparently possible similar electron-transfer pathways is actually functioning [7]. Homodimeric RCs must thus be studied in depth in order to compare them with heterodimeric RCs, if we are to understand the mechanisms of efficient energy conversion in photosynthesis among different types of RC.

Fds are low molecular weight iron–sulfur proteins functioning in a variety of electron-transfer reactions such as photosynthesis and nitrogen fixation. The iron–sulfur clusters bound in Fds are [4Fe–4S], [3Fe–4S] and [2Fe–2S] [8]. Fds from various organisms contain either one or two of these clusters. Fukuyama et al. [9] compared structures of Fds from various organisms, including several green sulfur bacteria, and discussed their evolutionary relationships. From *Chlorobium limicola*, Tanaka et al. sequenced two Fds of a 2[4Fe–4S] cluster type, namely FdI [10] and FdII [11], which are very acidic proteins consisting of 60 and 61 amino acid residues, respectively. Hase et al. [12] purified and sequenced a very acidic Fd from *Chlorobium thiosulfatophilum* strain Tassajara. They found it to be the only extremely acidic low-potential Fd found in this bacterium. The amino acid sequence of this Fd indicates that it belongs to a 2[4Fe–4S] cluster type. Functional properties of green sulfur bacterial Fds have been infrequently studied, however, because these Fds are assumed to be very unstable [13].

Although membrane preparations from green sulfur bacteria were shown to photoreduce $NADP^+$ [3], the activities reported for them were very low until recently. Miller et al. [14] reported a V_{max} of 23.1 μmol $NADP^+$ mg BChl a^{-1} h^{-1} in a preparation of membrane fragments from *Chlorobium vibrioforme*. More recently, Kjær and Scheller [15] reported that a chlorosome-less membrane preparation and a purified PS-C from *C. vibrioforme* photoreduced $NADP^+$ at rates of 333 and 110 μmol $NADP^+$ mg BChl a^{-1} h^{-1}, respectively.

The thermophilic green sulfur bacterium *Chlorobium tepidum* was discovered in 1991 [16], and has since proven useful because preparations from it are more stable at room temperature than those from mesophilic organisms. We purified RC complex from *C. tepidum* which was composed of five kinds of polypeptides, namely PscA–D and FMO protein, and showed that its photochemical activities are stable at room temperature [17,18,19].

We purified four Fd fractions from *C. tepidum*, and demonstrated that all of them efficiently accept electrons from purified *C. tepidum* PS-C.

2. Materials and methods

C. tepidum cells were grown for 12–17 h at 42.5°C, essentially according to Wahlund et al. [16], then harvested by continuous centrifugation ($10\,000\times g$) under anaerobic conditions, washed twice with a buffer (buffer A) containing 50 mM Tris–HCl (pH 7.8), 5 mM sodium ascorbate, 0.5 mM sodium dithionite and 5 mM dithiothreitol [17], and stored as pellets at −80°C. The frozen cells were then suspended in buffer A to which were added 1 mM phenylmethanesulfonyl fluoride, 1 mM *p*-aminobenzamidine–HCl, 1 mM 6-amino-*n*-caproic acid, and 5 U/ml DNase (Takara, Otsu), and disrupted by passing twice through a French pressure cell at 140 MPa. The suspension was centrifuged at $20\,000\times g$ for 20 min, and unbroken cells were removed as a precipitate. The green supernatant was further centrifuged at $160\,000\times g$ for 60 min, yielding pellets and a supernatant. Photochemically active PS-C complexes were prepared from the pellets under strictly anaerobic conditions as described in [19]. Briefly, buffers used in all steps from the extraction with Triton X-100 on contained 10% (v/v) glycerol, and the buffers for the subsequent DEAE-gel and hydroxyapatite chromatography contained no dithionite.

After ultracentrifugation, Fds were prepared from the supernatant under aerobic conditions. Powdered ammonium sulfate was added to the supernatant to 40% saturation, and the solution was gently stirred overnight at 4°C. The mixture was centrifuged at 20 000 × *g* for 20 min, and ammonium sulfate was added to the supernatant to 80% saturation. After gently stirring for more than 3 h, a light brown precipitate was collected by centrifugation at 20 000 × *g* for 20 min and stored at −80°C. The precipitate from 60 l of the culture (50–100 g wet cells) was suspended in 50 ml of 20 mM Tris–HCl buffer (pH 7.8) and dialyzed three times against 5 l of the same buffer for 3 h each time. The sample was then diluted to 150 ml with the same buffer, and applied to a DEAE-cellulose column (2.8 × 40 cm, DE23 Whatman) which had been equilibrated with the same buffer at 4°C. After washing the column with 50 mM Tris–HCl buffer (pH 7.8), the Fds were eluted with a 600 ml linear gradient of 0–800 mM NaCl in 50 mM Tris–HCl buffer (pH 7.8). Fd-rich fractions were located by acid-labile sulfide determination [20], concentrated by ultrafiltration (YM-3, Amicon) at 4°C and applied to a gel-permeation column (Sephacryl S-100 HR 26/60, Pharmacia) equilibrated with 50 mM Tris–HCl buffer (pH 7.8) containing 300 mM NaCl at 4°C. The column was eluted at a flow rate of 30 ml h^{-1}, and the Fds were eluted as light brown colored fractions. The combined Fd-rich fractions were mixed with an equal volume of saturated ammonium sulfate solution in 50 mM Tris–HCl buffer (pH 7.8), and applied to a hydrophobic column (Phenyl Superose 10/10, Pharmacia) equilibrated with 50 mM Tris–HCl buffer (pH 7.8) containing 2 M ammonium sulfate at room temperature. The column was washed with two column volumes of the equilibration medium, and the Fds were eluted as four major peaks (385 nm) with a 60 ml inverse linear gradient of ammonium sulfate of 2–0.8 M in 50 mM Tris–HCl (pH 7.8) followed by washing with the same buffer containing no ammonium sulfate. Each peak fraction was pooled separately, desalted with Ultrafree-4 (Millipore) and applied to an anion-exchange column (Mono Q 5/5, Pharmacia) equilibrated with 50 mM Tris–HCl (pH 7.8) containing 100 mM NaCl at room temperature. After washing the column with the same buffer, Fds were eluted with a 20 ml linear gradient of NaCl from 100 to 600 mM in the same Tris–HCl buffer. The purified Fds were stored either at 4°C or at −80°C until use. Spinach Fd and Fd-NADP$^+$ reductase (FNR [EC 1.18.1.2]) were prepared according to Shin et al. [21].

For light-induced NADP$^+$ photoreduction measurement, purified PS-C (2 nmol BChl *a*) was dissolved in a 1 ml mixture containing 50 mM Tris–HCl (pH 7.8), 50 mM NaCl, 5 mM sodium ascorbate, 0.1 mM 2,6-dichlorophenolindophenol, 0.1% Triton X-100, 0.1 mM NADP$^+$, 0.2 μM spinach FNR, 5 mM D-glucose, 1.25 U glucose-oxidase, 5×10^{-3} U catalase, 0.25% ethanol and Fd concentrations as indicated in Fig. 4. Reduction of NADP$^+$ ($\varepsilon_{340} = 6.2 \times 10^3$ M^{-1} cm^{-1}) was measured by absorbance changes at 340 nm with a spectrophotometer (Hitachi 557) equipped with an actinic tungsten light source and suitable filters. The cuvette was flushed with N_2 and kept anaerobic throughout the measurement.

BChl *a* concentration was determined as described by van der Rest et al. [22]. *C. tepidum* Fd concentrations were estimated from A_{385} assuming $\varepsilon_{385} = 30 \times 10^3$ M^{-1} cm^{-1} of *C. thiosulfatophilum* Fd [13]. Spinach Fd and spinach FNR concentrations were determined by assuming $\varepsilon_{420} = 9.7 \times 10^3$ M^{-1} cm^{-1} [13] and $\varepsilon_{456} = 10.74 \times 10^3$ M^{-1} cm^{-1} [21], respectively.

The N-terminal amino acid sequences of the purified Fds were determined by Edman degradation of the proteins, followed by sequence analysis with an Applied Biosystems Procise 494 cLC protein sequencer (Perkin-Elmer).

3. Results

3.1. Purification of Fds

When the fraction of the cell extract from *C. tepidum* precipitated between 40–80% saturated ammonium sulfate was further fractionated by DEAE-cellulose column chromatography, A_{280} was first eluted as a broad peak followed by a higher and sharper one (Fig. 1). Acid-labile sulfide was very rich in the second peak fraction, although it was also found in the first one. The second peak fraction contained Fds as will be described below, and its elution profile suggested that it was composed of several compo-

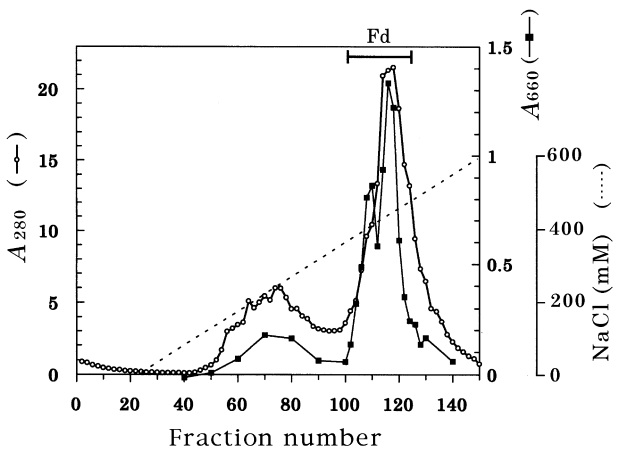

Fig. 1. Elution pattern of *C. tepidum* Fds from DEAE-cellulose column. Crude extracts precipitated between 40–80% saturated ammonium sulfate were fractionated by DEAE-cellulose (DE23, Whatman) open column chromatography. Column: 2.8×40 cm. Flow rate: 0.7 ml min^{-1}. Fraction volume: 5.25 ml. Temperature: 4°C. Aliquots of 0.1 ml from each fraction were analyzed for acid-labile sulfide (A_{660}). The upper bar indicates the fractions pooled for subsequent purification.

nents (Fig. 1). Protein determination by the Bradford method (Protein Assay, Bio-Rad) indicated that the second peak fraction contained a relatively low amount of protein (data not shown), suggesting that it was rich in nucleic acids. When the pooled second-peak fractions, rich in acid-labile sulfide, were further fractionated by Sephacryl S-100 gel-permeation chromatography, and monitored at A_{280}, they eluted in three peaks, the last one rich in acid-labile sulfide (data not shown). When the pooled last-peak fractions from the Sephacryl S-100 column were applied to a Phenyl Superose column and eluted with a decreasing concentration gradient of ammonium sulfate, monitoring at A_{385}, they eluted in four discernible peaks (A–D) with several minor ones (Fig. 2). The relative height of peak B was always high and that of peak A was low. Those of C and D varied among batches of culture. Generally speaking, the extracts from younger cultures (light, 12–14 h) were rich in C and those from older cultures (light, 15–17 h) were rich in D, but we did not study this further. The peak fractions A–D were separately pooled and purified by Mono Q ion-exchange column chromatography yielding Fds A–D. Their N-terminal amino acid sequences (15–25 residues) were determined (Table 1). The sequence of the first 15 N-terminal amino acid residues of FdA was new, and we later learned that it was identical to that deduced from the gene *fdx3* (Chung and Bryant, personal communication). The sequence of the first 25 residues of FdB was identical to that deduced from

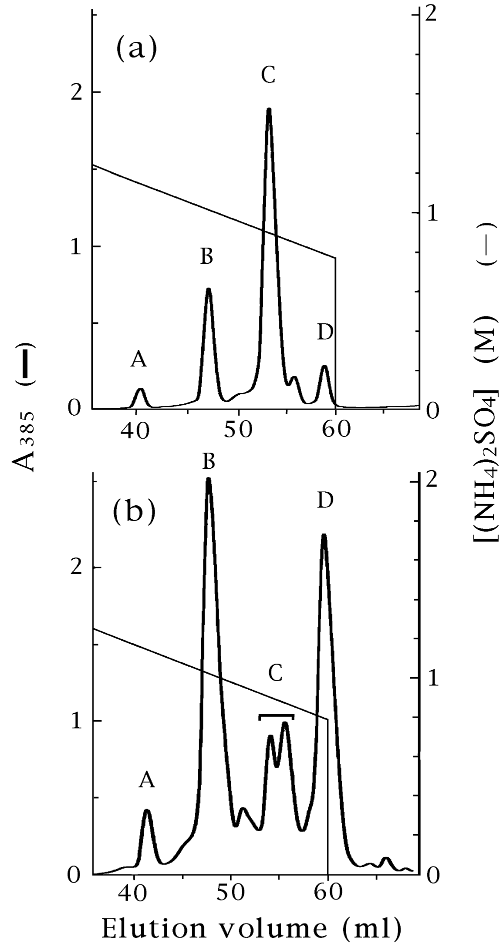

Fig. 2. Separation of Fds by hydrophobic chromatography. Acid-labile sulfide-rich fractions from the Sephacryl S-100 column were combined and purified using the FPLC system (Pharmacia). Column: Phenyl Superose 10/10 (Pharmacia). Flow rate: 0.5 ml min^{-1}. Fraction volume: 0.5 ml. Temperature: 23°C. Elution patterns of two different batches of cell extracts are shown. (a) Extracts from cells cultured for 12–14 h. (b) Extracts from cells cultured for 15–17 h. The total gradient solution volume was 60 ml. A–D indicate the fractions pooled for subsequent purification.

Table 1
N-terminal amino acid sequences of purified Fds

FdA	SLKITEECTFCAACE
FdB	AHRITDECTYCAACEPECPVSAISA
FdC,D	ALYITEECTYCGACE

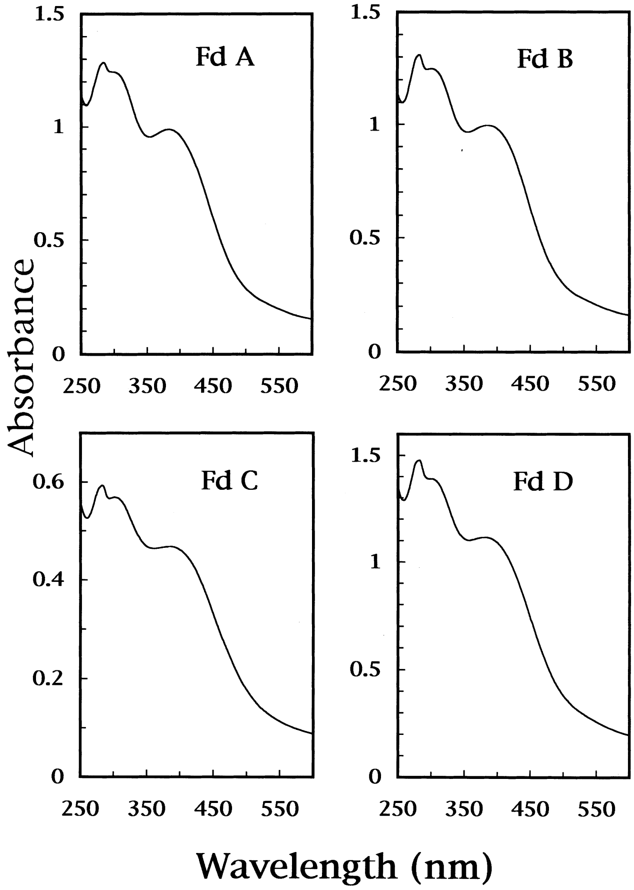

Fig. 3. Absorption spectra of purified *C. tepidum* FdA–D. Fds from the MonoQ column were further purified by passing through a gel-permeation column (Superdex 75 10/30, Pharmacia) equilibrated with 50 mM Tris–HCl buffer (pH 7.8) containing 300 mM NaCl at room temperature. This purification step marginally increased the value of A_{385}/A_{280} ratio (usually, a few percent). Fds were finally dissolved in 50 mM Tris–HCl (pH 7.8) and 400 mM NaCl, and the spectra were recorded at 23°C. The ratios A_{385}/A_{280} were: FdA, 0.78; FdB, 0.80; FdC, 0.80; FdD, 0.76.

Table 2
Kinetic constants of Fds

	V_{max} (μmol NADP$^+$ μmol BChl a^{-1} h^{-1})	K_m (μM)
C. tepidum FdA	258 ± 17	2.04 ± 0.27
C. tepidum FdB	304 ± 12	0.49 ± 0.06
C. tepidum FdC	226 ± 21	1.13 ± 0.25
C. tepidum FdD	242 ± 18	0.49 ± 0.12
Spinach Fd	183 ± 6.3	0.54 ± 0.06

Kinetic constants ± SE were analyzed from the data in Fig. 4 by a non-linear regression procedure.

the gene *fdx2* (Chung and Bryant, personal communication); these Fds will thus be referred to as FdIII and FdII. The sequence of the first 15 N-terminal amino acid residues of FdC was identical to that of FdD, and was also the same as that deduced from the gene *fdx1* (Chung and Bryant, personal communication).

The absorption spectra of FdA–D are typical of 2[4Fe–4S] Fds with absorption peaks at about 385 and 280 nm, with a shoulder at about 305 nm and with a trough at about 257 nm (Fig. 3). The A_{385}/A_{280} ratios of the purified FdA–D were between 0.76 and 0.80.

3.2. Photoreduction of Fds by purified PS-C

The activities of the purified *C. tepidum* FdA–D and spinach Fd were studied by measuring NADP$^+$ photoreduction at room temperature under strictly anaerobic conditions in a heterologous system con-

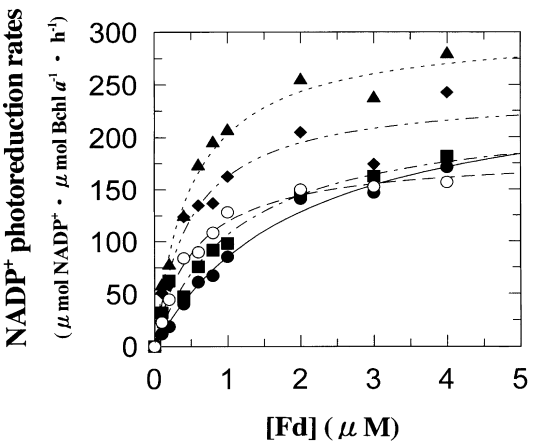

Fig. 4. Relationship between Fd concentration and the rate of NADP$^+$ photoreduction by *C. tepidum* PS-C with the deduced kinetic curves. The reaction mixture contained the indicated concentrations of FdA (●), FdB (▲), FdC (◆), FdD (■) from *C. tepidum* or Fd from spinach (○). *C. tepidum* Fds were purified up to the Mono Q step. Spinach FNR (0.2 μM) was substantially saturating under these conditions. The reaction mixture was illuminated at 23°C by yellow light at 1300 μmol m^{-2} s^{-1} for 1 min during which time A_{340} was continuously monitored. Kinetic constants (K_m and V_{max}) were analyzed by a non-linear regression procedure with the computer program ‘Grafit’ (version 3.0, 1992; Erithacus Software, London) (Table 2).

taining purified *C. tepidum* PS-C and an almost saturating amount of spinach FNR. The mixture was illuminated for 1 min, during which time photoreduction of $NADP^+$ was continuously monitored at 340 nm. The reaction proceeded almost linearly with time, all of FdA–D being highly active in supporting $NADP^+$ photoreduction (Fig. 4). The deduced V_{max} ranged from 226–304 μmol $NADP^+$ reduced μmol BChl a^{-1} h^{-1} (Table 2). There were some differences in V_{max} and affinity among these Fds. FdB showed the highest V_{max} and the highest affinity followed by FdD. It is of note that spinach Fd, a [2Fe–2S] cluster type, was fairly active, with a $K_m = 0.54$ μM lower than those of FdA and FdC in our assay system.

4. Discussion

Some microorganisms contain multiple forms of Fd. The purple photosynthetic bacterium *Rhodobacter capsulatus* contains at least six Fds: two 2[4Fe–4S], one ([3Fe–4S]+[4Fe–4S]) and three [2Fe–2S] cluster types [23]. In green sulfur bacteria, two Fds were found in *C. limicola* [10,11] and only one in *C. thiosulfatophilum* [12]. More recently, Yoon et al. [24] briefly reported the occurrence of two Fds in *C. tepidum*, which were active in the pyruvate synthase reaction. We found that *C. tepidum* contains at least three Fds with characteristic absorption spectra of [4Fe–4S] cluster type Fds (Fig. 3). This was also supported by the amino acid sequences of these Fds, deduced from the respective *fdx* genes (Chung and Bryant, personal communication). These Fds seem to be very acidic because they stain poorly with Coomassie Brilliant Blue (data not shown) and require high NaCl concentrations to be eluted from a DEAE-cellulose column (Fig. 1). The A_{385}/A_{280} ratios of 0.76–0.80 of these Fds (Fig. 3) are similar to those of other Fds from green sulfur bacteria: A_{390}/A_{280} ratio of 0.77 for *C. limicola* FdI [10], and A_{385}/A_{280} ratio of 0.71 for *C. thiosulfatophilum* Fd [25].

The sequence of the first 15 N-terminal amino acid residues of FdC was identical to that of FdD, although they behaved differently on Phenyl-Superose column chromatography. Neither a freeze–thaw cycle, nor heating these Fds for 40 min at 50°C changed their chromatographic behaviors. We do not know at present if FdC and FdD are isoforms of the same Fd, if either of them is modified, or if they are actually different proteins.

C. thiosulfatophilum Fd was reported to be very unstable [3,13]. The latter authors [13] reported that the A_{385}/A_{280} ratio of the purified Fd dropped from 0.71 to 0.60 when stored for 5 h at 4°C in air and recommended that the purification be performed rapidly, with the total time not to exceed 3 days. We found that when purified FdA–D from *C. tepidum* were stored at 4°C under air, their A_{385} values decreased by 10–15% after 6 days (data not shown), indicating that they are not extremely unstable. When these Fds were stored frozen at −80°C under aerobic conditions, their A_{385} values did not decrease after one month. Our purification procedures normally lasted a week, and were carried out in air. Yet we were able to prepare substantial amounts of these Fds. It will be interesting to compare the amino acid sequence among green sulfur bacterial Fds and to find the key factors which confer stability to *C. tepidum* Fds.

Although it was shown more than 30 years ago that membranes from green sulfur bacteria can directly reduce Fd [3], the reported activities of membrane preparations from these organisms were very low. Recently, however, Kjær and Scheller [15], using *Clostridium pasteurianum* Fd and spinach FNR, reported that a membrane preparation, and a PS-C preparation consisting of six kinds of polypeptides from *C. vibrioforme*, photoreduced $NADP^+$ at 331 and 110 (maximally 150) μmol $NADP^+$ mg BChl a^{-1} h^{-1}, respectively. The former activity was more than 10 times higher, and the latter 4–5 times higher on a BChl *a* basis than any previously reported with membrane preparations. Our PS-C preparation from *C. tepidum* contained five types of polypeptides, and was highly active in $NADP^+$ photoreduction at room temperature in the presence of Fd either from this organism or from spinach and an almost saturating amount of FNR from spinach. Under these conditions, the K_m values obtained are principally determined by the reaction between PS-C and Fds. The highest activity was obtained with FdB, at 304 μmol $NADP^+$ μmol BChl a^{-1} h^{-1}, more than twice that reported with purified PS-C from *C. vibrioforme* [15]. Spinach Fd was fairly active with V_{max} of about 60–80% of those of FdA–D (Fig. 4, Table 2). Kjær and Scheller [15] reported that Fd from barley or spinach was about one-third as active as that from *C. pasteurianum* in $NADP^+$ photoreduction by purified PS-C preparation from *C. vibrioforme*. Such large differences in activity among *C. tepidum* Fds and spinach Fd were not found in *C. tepidum* PS-C. The affinity reported for the photoreduction of Fd in membrane preparations of *C. thiosulfatophilum*, as assayed by a pyruvate synthase reaction, was low, the reaction not being saturated at 100 μg Fd in 3 ml of the reaction mixture or at about 5 μM Fd [3]. We found that the affinity of *C. tepidum* PS-C particles for Fds is relatively high, with K_m of 0.49–2.0 μM.

PSI particles from spinach contain 14–15 kinds of polypeptide [26], while our highly active PS-C preparation contained only five kinds [17]: PscA, a core; PscB (31 and 24 kDa, in the apparent and the actual molecular mass, respectively), the terminal iron–sulfur protein [5]; PscC (24 and 23 kDa), bound cyt *c*551; PscD (18 and 16.5 kDa, respectively), of unknown function [27]; and FMO protein, an antennae. PS-C preparation from *C. vibrioforme* contained six kinds of polypeptides, five corresponding to those of *C. tepidum* and an additional 9 kDa polypeptide of unknown function [15]. Our results indicate that the PS-C preparation from *C. tepidum* catalyzes efficient $NADP^+$ photoreduction without a counterpart of the 9 kDa polypeptide found in *C. vibrioforme* PS-C.

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