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Reduction of ferredoxins by photosynthetic reaction center complex from the green sulfur bacterium *Chlorobium tepidum*

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1. Introduction

Green sulfur bacteria are photoautotrophs that use inorganic sulfur compounds as electron donors under anaerobic conditions. Green sulfur bacterial reaction centers (PS-Cs) belong to an iron-sulfur type which also includes PSI and heliobacterial RCs. PSI and PS-C have low potential iron-sulfur clusters as terminal electron accepters and can reduce ferredoxin (Fd) directly.

Fds are low molecular weight iron-sulfur proteins functioning in various electron-transfer reactions such as $NAD(P)^+$ reduction in photosynthesis, nitrogen fixation, and nitrite reduction. From several green sulfur bacteria, 2[4Fe-4S] cluster type Fds were purified and their amino acid sequences were determined: two Fds from *Chlorobium limicola* (Tanaka et al.), and one from *C. thiosulfatophilum* strain *Tassajara* (Hase et al.). The amino acid sequences of these Fds are highly conserved among these green sulfur bacteria. Functional properties of green sulfur bacterial Fds have been infrequently studied, however, because these Fds are assumed to be very unstable to oxygen (Buchanan et al.).

The thermophilic green sulfur bacterium *Chlorobium tepidum* was discovered in 1991, and has since proven useful because preparations from it are stable at room temperature and the whole genome was sequenced at The Institute for Genomic Research. We purified PS-C complex from *C. tepidum* that was composed of 5 kinds of polypeptides, namely Psc A-D and FMO protein, and showed that its photochemical activities are stable at room temperature. We purified four Fd fractions from *C. tepidum*, and demonstrated that all of them efficiently accept electrons from purified PS-C of the same organism.

2. Materials and Methods

C. tepidum cells were grown at 42.5 ºC, according to Wahlund et al., harvested by centrifugation under anaerobic conditions and stored as pellets at –80 ºC. The frozen cells were suspended and disrupted by passing twice through a French pressure cell at 140 MPa. The suspension was ultracentrifuged, yielding pellets and a supernatant. PS-C complexes were prepared from the pellets under strictly anaerobic conditions. Fds were prepared from the supernatant under aerobic conditions. The precipitate obtained between 40% and 80% saturating ammonium sulfate was dialyzed and the dialysate was applied to a DEAE-cellulose column (DE23, Whatman). The Fds were eluted with a linear gradient of 0 to 800 mM NaCl. Fd-rich fractions located by acid-labile sulfide determination were concentrated by ultrafiltration and applied to a gel-permeation column (Sephacryl S-100 HR 26/60, Pharmacia). The Fd-rich fractions were made to 2M in ammonium sulfate and applied to a hydrophobic column (Phenyl Superose 10/10, Pharmacia) and were eluted with an inverse linear gradient of ammonium sulfate yielding four major peaks in A_{385}. Each peak fraction was pooled separately, desalted and further purified with anion exchange chromatography (Mono Q, Pharmacia).

For light-induced $NADP^+$ photoreduction measurements, purified PS-C was dissolved in a 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 units glucose-oxidase, 5×10^{-3} units catalase, 0.25% ethanol, and Fd as indicated. Reduction of $NADP^+$ was measured by absorbance changes at 340 nm in a stoppered cuvette which was flushed with N_2 and kept anaerobic throughout the measurement.

3. Results and Discussion

The elution profile from a Phenyl Superose column showed 4 discernible peaks of A_{385} (A-D) with several minor ones. The relative heights of peak B and D were always high, that of peak A was low, and that of peak C varied among batches of culture. Roughly speaking, the extracts from younger cultures were high and those from older cultures were relatively low in peak C. The N-terminal amino acid sequences (15-25 residues) of these four Fds were determined. The sequence of the first 15 N-terminal amino acid residues of FdA corresponds and the first 25 residues of FdB to those deduced from the genes *fdx*3 and *fdx*2, respectively (Chung and Bryant, personal communication). 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 *fdx*1 (Chung and Bryant, personal communication). This is an unexpected result, and 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. Recently, Yoon et al. briefly reported the occurrence of two Fds in *C. tepidum*, which were active in the pyruvate Fd oxidoreductase reaction. The correspondences of these Fds to those described here are not certain. Vassilieva et al.

reported [2Fe-2S] type iron-sulfur proteins in *C. tepidum*, but they seem to be localized in chlorosome envelope.

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

C. thiosulfatophilum Fd was reported to be extremely sensitive to oxygen. In contrast with this, when purified FdA-D from *C. tepidum* were stored at 4C under air, their A_{385} values decreased by 10–15% after 6 days, indicating that they are not extremely unstable (Fig. 1). When these Fds were stored frozen at –80C 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

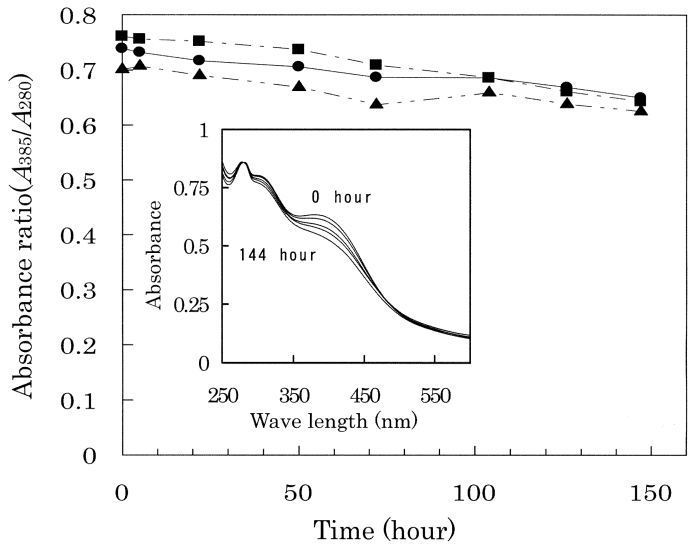

Fig. 1. Stability of purified Fds from *C. tepidum*. Fd A(▲), Fd B (■), and Fd D (●) were dissolved in 50 mM Tris-HCl (pH 7.8) buffer containing 300 mM NaCl and incubated for the time indicated under aerobic and dark conditions at 4°C. The insert indicates the changes of absorption spectrum of Fd B.

these Fds.

Although it was shown more than 30 years ago that membranes from green sulfur bacteria could directly reduce Fd, the reported activities of membrane preparations from these organisms were very low. Recently, however, Kjær and Scheller, using *Clostridium pasteurianum* Fd and spinach FNR, reported that a membrane preparation, and a PS-C preparation consisting of 6 kinds of polypeptides from *C. vibrioforme*, photoreduced $NADP^+$ at 331 and 110 (maximally 150) μmol $NADP^+$ mg BChl a^{-1} h^{-1}, respectively. Our PS-C preparation from *C. tepidum* contained 5 kinds 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 (Fig. 2). The highest activity was obtained with FdB (756 μmol $NADP^+$ μmol BChl a^{-1} h^{-1}). Spinach Fd was fairly active with V_{max} of about 60-105 % of those of FdA, B and D.

Evans et al. reported that the affinity for the photoreduction of Fd in membrane preparations of *C. thiosulfatophilum* was low, the reaction not being saturated at 100 μg Fd in 3 ml of the reaction mixture or at about 5 μM Fd. We found that the affinity of *C.*

tepidum PS-C particles for Fds is relatively high, with K_m of 0.49-0.6 μM.

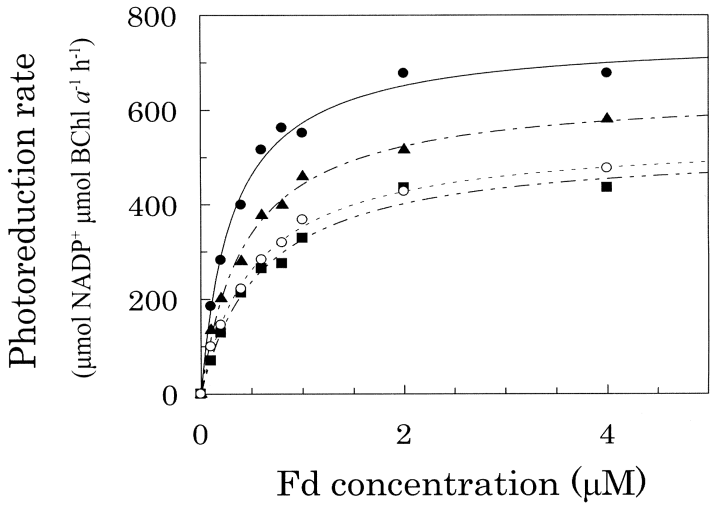

Fig. 2. Relationship between Fd concentration and the rate of $NADP^+$ photoreduction by *C. tepidum* PS-C with the deduced kinetic curves. The reaction mixture contained the indicated concentrations of FdA (■), FdB (●), FdD (▲) from *C. tepidum* or Fd from spinach (○). The reaction mixture was illuminated at 23°C by yellow light at 1,300 μmol $m^{-2}s^{-1}$ for 1 min during which time A_{340} was continuously monitored.

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