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ISOLATION AND PROPERTIES OF RUBREDOXIN FROM THE PHOTOSYNTHETIC GREEN SULFUR BACTERIA

T. E. MEYER, J. J. SHARP AND R. G. BARTSCH
Department of Chemistry, University of California, San Diego, La Jolla, Calif. 92037 (U.S.A.)
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SUMMARY

Rubredoxin with properties like those of clostridial rubredoxin has been isolated from two species of green sulfur photosynthetic bacteria, *Chlorobium thiosulfatophilum* and *Choloropseudomonas ethylicum*. Rubredoxin was not detected in the several purple sulfur and non-sulfur photosynthetic bacteria tested. No indication of the role of rubredoxin in photosynthetic bacteria is yet apparent.

Rubredoxin was first isolated from *Clostridium pasteurianum* and characterized as a non-heme iron protein by LOVENBERG AND SOBEL[1]. Rubredoxin is a protein having molecular weight 6000, one iron atom, and no inorganic sulfide[1]. The absorption spectrum of the oxidized form has maxima at 280, 380 and 490 nm and is completely bleached on reduction[1]. The oxidation–reduction potential is −57 mV at pH 7.0 (ref. 1). Rubredoxins have been found in several species of bacteria: *Clostridium sticklandii*[2], *Desulfovibrio gigas*[3], *Desulfovibrio desulfuricans*[4], *Peptostreptococcus elsdenii*[5], *Pseudomonas oleovorans*[6], *Micrococcus aerogenes*[7], and *Micrococcus lactolyticus*[8]. The amino acid sequences of *Micrococcus aerogenes*[9] and *Peptostreptococcus elsdenii*[10] rubredoxins have been elucidated. *Pseudomonas oleovorans* rubredoxin is exceptional in having a size twice that of other rubredoxins and in having twice the iron content[11]. With the possible exception of *Pseudomonas oleovorans*, all the above organisms contain a bacterial ferredoxin. Whereas ferredoxin has been found in all photosynthetic bacteria examined, rubredoxin has not previously been reported.

Chlorobium thiosulfatophilum PM was grown in Larsen's medium[12] with added sodium acetate[13], and *Chloropseudomonas ethylicum* 2K was grown in a modified Kondrat'eva medium[12]. Cells in 20% (w/v) suspension in 0.1 M Tris–HCl, pH 7.3, were disrupted in the Sorvall-Ribi cell fractionator operated at 20000 lbs/inch^2 and 20°. The broken cell suspensions were centrifuged in the Servall SS-34 rotor at 30000 $\times g$ for 10 min for Chlorobium and 40 min for Chloropseudomonas. The Servall supernatant solution was centrifuged in the Spinco Type 42 rotor at 205700 $\times g_{max}$ for 2 h. Ferredoxin and rubredoxin were adsorbed from the Spinco supernatant solution on a column of DEAE-cellulose (Selectacel Standard, Brown Co.) and the column was washed with 0.1 M Tris–HCl, pH 7.3. The proteins were eluted from the column with 0.5 M NaCl in 0.1 M Tris–HCl, pH 7.3, and desalted on a G-25 Sephadex column with buffer change to 0.1 M Tris–HCl, pH 7.3. The proteins were again adsorbed on

DEAE-cellulose and chromatographed with a stepwise NaCl gradient in 0.1 M Tris buffer. The purple-colored rubredoxin separated from the green ferredoxin and was eluted with 0.10–0.12 M NaCl in the Tris buffer. Ferredoxin was eluted with 0.25–0.30 M NaCl in the Tris buffer.

The yield of crude ferredoxin from *Chlorobium thiosulfatophilum* was 15 μmoles/kg wet wt. cells and from *Chloropseudomonas ethylicum* it was 90 μmoles/kg wet wt. cells. The absorptivity at 390 nm was assumed to be 20 $mM^{-1} \cdot cm^{-1}$, the same as reported for *Clostridium pasteurianum* ferredoxin[14]. The yield of crude rubredoxin from *Chlorobium thiosulfatophilum* was 4 μmoles/kg wet wt. cells and from *Chloropseudomonas ethylicum* it was 12 μmoles/kg wet wt. cells. The absorptivity at 490 nm was assumed to be 8.85 $mM^{-1} \cdot cm^{-1}$, the same as that for *Clostridium pasteurianum* rubredoxin[1]. *Chlorobium thiosulfatophilum* rubredoxin was further purified by rechromatography on DEAE-cellulose until the absorbance ratio $A_{280\ nm}/A_{490\ nm}$ was constant. The best ratio obtained was 2.65. The purified rubredoxin had absorption maxima at 280, 372 and 490 nm in the oxidized state and was completely bleached on reduction (Fig. 1). The reduced form was rapidly reoxidized in air.

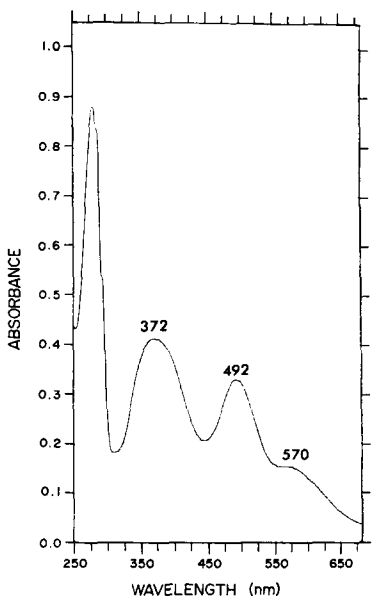

Fig. 1. Absorption spectrum of oxidized *Chlorobium thiosulfatophilum* rubredoxin in 50 mM potassium phosphate buffer, pH 7.0. Addition of solid $Na_2S_2O_4$ to the mixture completely bleached the visible spectrum.

The electron paramagnetic resonance spectrum of oxidized rubredoxin at pH 7.0 and 80°K showed an 18 gauss wide resonance at $g = 4.3$, similar to that observed for other rubredoxins[5,7].

The oxidation–reduction potential of rubredoxin (48 μM) was calculated from the relative changes in light absorption of rubredoxin at 490 nm and of indigo tetrasulfonate (8 μM) at 600 nm when titrated anaerobically with sodium dithionite. The redox potential of Chlorobium rubredoxin was found to be —61 mV at pH 7.0 (0.1 M

phosphate buffer) and 23°. For indigo tetrasulfonate $E_{m,7} = -46$ mV (ref. 15) was assumed.

The molecular size of the rubredoxins was measured by comparison with horse heart cytochrome *c* (mol. wt. 12400), sperm whale myoglobin (mol. wt. 17400), *Chromatium vinosum* ATCC 17899 flavocytochrome *c* (mol. wt. 72000), and chymotrypsinogen (mol. wt. 25000) on a 1.4 cm × 50 cm column of Sephadex G-75-F, equilibrated with 0.1 M Tris–HCl, pH 7.3, plus 0.5 M NaCl (ref. 16). The size of *Chlorobium thiosulfatophilum* rubredoxin, expressed in molecular weight units, was found to be 7400 and that of *Chloropseudomonas ethylicum* rubredoxin to be 7800. The actual molecular weight is expected to be smaller than this, probably as low as 6000, as with most other rubredoxins.

The amino acid composition of *Chlorobium thiosulfatophilum* rubredoxin was determined on the Beckman Spinco amino acid analyzer following 48 h anaerobic hydrolysis in constant boiling HCl at 100°. The amino acid composition obtained was: Asp_{10} Thr_1 Ser_3 Glu_6 Pro_7 Gly_5 Ala_2 $Cys_{(4)}$ Val_4 Met_1 Ile_1 Leu_3 Tyr_2 Phe_3 His_0 Lys_2 Arg_0 $Trp_{(1)}$, total 55 residues. Only two cysteines were recovered, but judging by the cysteine recovery from other proteins treated in the same way, four cysteine residues are assumed. Tryptophan was not measured, but is assumed to be one residue in analogy with other rubredoxins[9,10]. From these results, the formula weight, 6000, may be calculated. The composition is more similar to *Micrococcus aerogenes*[9] than to *Clostridium pasteurianum* rubredoxin[1].

Rubredoxin was found in the green photosynthetic bacteria, but not in *Chromatium vinosum* ATCC 17899, *Rhodospirillum rubrum* ATCC 11170, or *Rhodopseudomonas palustris* ATCC 17007, even though ferredoxin has been found in all strains of photosynthetic bacteria examined. Ferredoxin had been found in nearly all bacteria in which rubredoxin had been discovered, but it is now sufficiently clear that there is no relationship between the distribution of the two types of iron proteins.

The specific electron transfer function of rubredoxin may be the same in most bacteria, in which it is found, in the light of close structural similarities between most members of this protein class. However, the specific function of rubredoxin has been worked out only in *Pseudomonas oleovorans*, where the protein acts as the reducing agent for a mixed function oxidase[6], a role which is excluded in all the anaerobic bacteria which possess rubredoxin.

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