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PROPERTIES AND REGULATION OF SYNTHESIS OF TWO FERREDOXINS FROM *RHODOPSEUDOMONAS CAPSULATA*

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Two ferredoxins from nitrogen-fixing cells of the phototrophic bacterium *Rhodopseudomonas capsulata*, strain B10, are purified to a homogeneous state and characterized. The molecular mass of ferredoxin I is about 12 kDa and that of ferredoxin II, 18 kDa. Ferredoxin I contains 8 Fe^{2+} and 8 S^{2-}; ferredoxin II has 4 Fe^{2+} and 4 S^{2-} per molecule. The redox potential of ferredoxin I is about −270 mV and that of ferredoxin II −419 mV. Ferredoxin I is more labile to the action of O_2, O_2^-, H_2O_2 and heating. The ferredoxins are also different in their absorption and EPR spectra, amino acid composition and electron-transfer activity to *Rps. capsulata* nitrogenase: both C_2H_2 reduction and H_2 evolution by *Rps. capsulata* nitrogenase proceed faster in the presence of ferredoxin I than in case of ferredoxin II. Synthesis of ferredoxin I takes place only in *Rps. capsulata* nitrogen-fixing cells grown in light under anaerobic conditions whereas ferredoxin II formation does not depend on the source of nitrogen or the growth medium, though the amount of ferredoxin II varies with the growth conditions. Its highest level has been found in the cells grown in lactate-limited medium in the presence of CO_2 and light or in the presence of glutamate in darkness under anaerobic conditions.

Introduction

Two ferredoxins with a number of different properties have been obtained from *Azotobacter vinelandii* [1], *Bacillus polymyxa* [2], *Xanthobacter flavus (= Mycobacterium flavum)* [3], *Corynebacterium autotrophicum* [4], *Desulfovibrio gigas* [5], *D. desulfuricans* [6], *D. africanus* [7], *Rhodospirillum rubrum* [8] and *Spirulina maxima* [9]. There are very limited data on the in vitro reaction between ferredoxins and nitrogenase in N_2-fixing bacteria. Usually, the rate of acetylene reduction is lower in the presence of reduced ferredoxins than in the presence of dithionite [10]. Only in *Rhizobium japonicum* has it been shown recently that acetylene reduction proceeds at higher rate in the presence of reduced ferredoxin as an electron donor than in the presence of dithionite [11]. In vivo, both ferredoxins from nitrogen-fixing bacteria are assumed to be capable of acting as electron donor for nitrogenase [12].

In *R. rubrum* ferredoxin I has been shown to be synthesized only in illuminated cells [8]. Ferredoxin I is more active as an electron carrier to nitrogenase [13] than ferredoxin II. Metronidazole, an inhibitor of ferredoxin-dependent reactions, has been demonstrated to inhibit the activity of nitrogenase from *Rhodopseudomonas capsulata* in vivo [14]. However, unlike *R. rubrum Rps. capsulata* shows only one ferredoxin which is similar in its properties to ferredoxin I of *R. rubrum* [15–17].

In this paper the isolation, biosynthesis, possi-

Abbreviation: DCIP, 2,6-dichlorophenolindophenol.

ble functions and some molecular properties of two ferredoxins of the purple non-sulphur bacterium *Rps. capsulata* are described.

Materials and Methods

Bacterial culture. Cells of *Rps. capsulata*, strain B10, were grown in the medium described by Ormerod et al. [18] with lactate in a continuous fermentor for phototrophic microorganisms [19]. The cells were grown under anaerobic conditions in light or under microaerobic conditions in the dark with excess $(NH_4)_2SO_4$ (10 mM) or its limiting amount (4 mM). In some cases the cells cultivated under anaerobic conditions in light with excess of ammonium and lactate limitation (9 mM) in the presence or absence of CO_2 were used. When the cells were grown in the dark under anaerobic conditions, glucose (30 mM) and dimethyl sulphoxide (60 mM) were supplemented to the medium of Ormerod et al. [18] instead of lactate as described by Madigan et al. [20]. The cells were harvested in a continuous-flow centrifuge and stored in liquid nitrogen.

Purification of ferredoxins and nitrogenase. All operations of purifications were carried out under anaerobic conditions. The buffer solutions were preliminarily degassed, bubbled with argon gas and the chromatographic columns were washed by several volumes of anaerobic buffer. The eluted fractions were collected in vessels covered with rubber stoppers and filled with argon.

The cells (350 g wet weight) were resuspended in 700 ml of 0.1 M Tris-acetate buffer (pH 8.1) and sonicated for 5 min (22 kHz; 4°C) with a UZDN-1 disintegrator (U.S.S.R.). The crude extract was centrifuged ($30\,000 \times g$; 60 min; 4°C) and the supernatant was applied to a 2.5×20 cm DEAE-cellulose DE_{32} column, equilibrated with 0.1 M NaCl in 50 mM Tris-acetate buffer (pH 7.5). The column was washed with several volumes of 0.1 M NaCl in the same buffer until colourless eluate was obtained. Nitrogenase and ferredoxins were separated with 90 mM $MgCl_2$ in the buffer. Nitrogenase eluated first from the column; this fraction did not contain ferredoxins. Such preparations was used for determination of the activity of ferredoxins as electron carriers in a system containing illuminated pea chloroplasts.

For further purification the ferredoxin fraction was chromatographed in 80-mg portions on a 3.5×100 cm Sephadex G-75 (superfine) column, equilibrated with 0.1 M NaCl in 50 mM Tris-acetate buffer, pH 7.5 (Table I). At this stage of purification two forms of ferredoxin were separated and ferredoxin II was the first to be eluated from the column. Ferredoxins were collected separately and applied to a 1×7 cm DEAE-cellulose DE_{52} column, equilibrated with 0.1 M NaCl in the buffer. Ferredoxins II was eluated from the column by a linear gradient of NaCl (0.1–0.5 M), ferredoxin I by a gradient of 0.1–0.3 M NaCl in the buffer. Ferredoxin II was collected from the column at an NaCl concentration of about 0.3 M ferredoxin I at about 0.2 M NaCl. At this stage the protein was judged to be pure both from polyacrylamide gel electrophoresis and from N-terminal amino acid analysis. The yield of ferredoxin I from *Rps. capsulata* nitrogen-fixing cells was 4–5-times as high as that of ferredoxin II.

For some experiments partially purified preparations of ferredoxins were obtained by chromatography of the soluble fraction on a DEAE-cellulose column. The protein fraction containing ferredoxin was eluated by 90 mM $MgCl_2$ in the buffer. The preparations of ferredoxins and nitrogenase were stored in liquid nitrogen.

Analytical procedures. The relative molecular mass of ferredoxins was determined by gel filtration on a 1.0×40 cm column of Sephadex G-75 (fine). Spinach ferredoxin (10 500), cytochrome *c* from horse heart (13 000), chymotrypsinogen A (25 000), deoxyribonuclease I (31 000) and ovalbumin (45 000) were used as marker proteins.

The analytical disc gel electrophoresis was carried out in 15% polyacrylamide gel [21].

Absorption spectra were recorded on a Specord UV-VIS spectrophotometer (G.D.R.) and EPR spectra on a 3 cm superheterodyne spectrometer RE-1302 (U.S.S.R.).

Amino acid composition of ferredoxins was determined by a Durrum D-500 amino acid analyzer (U.S.A.). The tryptophan content of ferredoxin was determined by the method of Edelhoch [22]. N-terminal amino acids were identified by the dansyl chloride procedure [23].

Iron was estimated by atomic absorption spectrometry using a Perkin-Elmer 503 spectrometer

TABLE I

PURIFICATION OF FERREDOXINS OF *RPS. CAPSULATA*

Ferredoxin activity was estimated by the rate of electron transfer to *Rps. capsulata* nitrogenase in the system with chloroplasts (see Materials and methods). 1 unit is defined as 1 nmol C_2H_4 formed/min.

Purification steps	Volume (ml)	Protein (mg)	Total activity (U)	Specific activity (U/mg protein)
Crude extract	700	32200	12880	0.4
Chromatography on DEAE-cellulose DE_{32} (elution by 90 mM $MgCl_2$)	70	280	109200	390
Gel filtration on G-75				
ferredoxin I	120	102	82072	805
ferredoxin II	126	43	13320	310
Chromatography on DEAE-cellulose DE_{52} (elution by NaCl linear gradient)				
ferredoxin I	17	33	66311	2009
ferredoxin II	6	11	8658	787

(Sweden). The acid-labile sulphur was determined with *N,N'*-dimethylphenylenediamine [24].

The redox potential of ferredoxins was determined by potentiometric titration of samples in a cell with a platinum electrode and calomel reference electrode. The samples of ferredoxins (25–35 μM) in 0.1 M Tris-acetate buffer (pH 7.5) were reduced by adding 4–5 μl of 10 mM dithionite solution. The extent of ferredoxin oxidation (reduction) was followed by changes in the absorption spectra.

The activity of ferredoxins as electron carriers to *Rps. capsulata* nitrogenase was measured in a reaction mixture containing in a final volume of 1 ml the following components: 50 mM Hepes-K^+ buffer (pH 7.5), 30 mM sodium creatine phosphate, 0.2 mg creatine phosphokinase, 5 mM ATP, 5 mM $MgCl_2$, 13 mM sodium ascorbate, 0.03 mM 2,6-dichlorophenolindophenol, 1 mg nitrogenase, pea chloroplasts equivalent to 0.3 mg chlorophyll *a* and ferredoxins as indicated. The gas phase was as follows: 90% Ar + 10% C_2H_2, if the nitrogenase activity was determined by the reaction of C_2H_2 reduction or 100% Ar for H_2 evolution. The reaction vessels were incubated in a thermostatically controlled shaker under light (30°C; $7 \cdot 10^4$ erg/cm^2 per s). After 15 min the reaction was terminated by trichloroacetic acid (up to 5%) and the amount of ethylene or H_2 was measured by gas chromatography [25].

The preparations of superoxide dismutase from *Rps. capsulata* were partially purified by the method described in Ref. 26. Superoxide dismutase preparations with a specific activity of 50 U/mg protein were used.

Chloroplasts from pea leaves were isolated by the method of West and Wischich [27]. To avoid O_2 evolution due to the action of Photosystem II, the latter was inactivated by heating (55°C; 5 min).

The amount of chlorophyll was determined by the method of Arnon [28] and that of protein by the method of Lowry et al. [29] with bovine serum albumin as a standard.

Results

Absorption spectra and extinction coefficients

The spectra of the oxidized ferredoxin I has

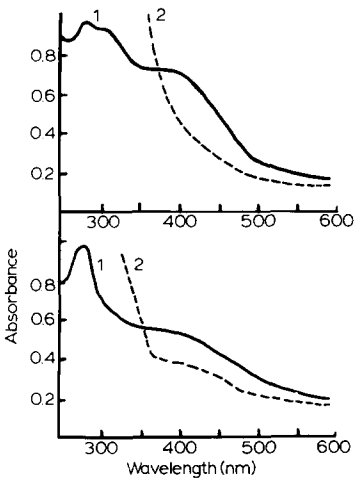

Fig. 1. Absorption spectra of *Rps. capsulata* ferredoxins in oxidized (———) and dithionite-reduced (- - - - - -) states. Cuvettes contained (1 cm light pathway): ferredoxin I (25 μM) in 50 mM Tris-acetate buffer (pH 7.5)+0.2 M NaCl or ferredoxin II (44 μM) in 50 mM Tris-acetate buffer (pH 7.5)+0.3 M NaCl.

maxima at 280 and 380 nm, and a shoulder at 310 nm (Fig. 1) which is characteristic of bacterial ferredoxins, type 2 [4Fe-4S]. Its millimolar extinction coefficient is $\epsilon_{380} = 29$ and absorbance ratio $A_{380}/A_{280} = 0.78$, which is also typical of this type of ferredoxin. After ferredoxin reduction with dithionite a decrease in absorption takes place in the 380–600 nm region.

Maxima at 280 and 380 nm are typical of the ferredoxin II absorption spectrum (Fig. 1). The latter has a millimolar coefficient of extinction $\epsilon_{380} = 12.3$, and $A_{380}/A_{280} = 0.55$ (Table II). Such absorption spectra are characteristic of ferredoxins with one cluster [4Fe-4S].

Molecular mass

The relative molecular mass of ferredoxins determined by gel filtration in a Sephadex G-75 column was shown to be 12000 for ferredoxin I, and 18000 for ferredoxin II. The minimum molecular mass of ferredoxins calculated for the amino acid, iron and sulphur contents, was 11800 for ferredoxin I, and 17800 for ferredoxin II (Tables II and III).

Iron and acid-labile sulphur content

Iron content in ferredoxins was found to be 8 Fe^{2+} (ferredoxin I) and 4 Fe^{2+} (ferredoxin II) per mol and sulphur content 8.5 S^{2-} (ferredoxin I) and 4.6 S^{2-} (ferredoxin II) mol per mol ferredoxin (Table II).

TABLE II

PROPERTIES OF FERREDOXINS OF *RPS. CAPSULATA*

Properties	Ferredoxin I	Ferredoxin II
Molecular mass	12000 ±1000	18000 ±1000
Content (mol/mol protein)		
iron	8.0 ± 0.6	4.0 ± 0.5
S^{2-}	8.5 ± 0.6	4.6 ± 0.5
Extinction coefficient ($mM^{-1} \cdot cm^{-1}$)	$\epsilon_{280} = 38.0$ $\epsilon_{380} = 29.0$	$\epsilon_{280} = 22.3$ $\epsilon_{380} = 12.3$
A_{380}/A_{280}	0.78± 0.03	0.52± 0.03
Oxidation-reduction potential (pH 7.5) (mV)	−270 ± 20	−419± 10
N-terminal amino acid	alanine	threonine
Activity as electron carrier to *Rps. capsulata* nitrogenase (nmol/min per mg protein)		
reduction of C_2H_2	2100 ± 100	840 ± 100
evolution of H_2	3000 ± 100	1830 ± 100
Yield (mg/250 g frozen cells)	25 – 30	6 – 7

Amino acid composition

Arginine, phenylalanine and tryptophan were not found in ferredoxin I (Table III). All common amino acids besides tyrosine were shown to be typical of ferredoxin II, but unlike ferredoxin I it has only about four cysteine residues. This also suggested the presence of only a single [4Fe-4S] cluster in ferredoxin II. The content of acidic amino acids in ferredoxin II was higher in comparison with that of ferredoxin I which was apparently the reason for its stronger interaction with DEAE-cellulose. N-terminal amino acid analysis showed alanine in ferredoxin I and threonine in ferredoxin II. The presence of a single N-termi-

TABLE III

AMINO ACID COMPOSITION OF FERREDOXINS OF *RPS. CAPSULATA*

Amino acid analysis was carried out after hydrolysis of preparations in vacuum (6 M HCl, 105°C, 34 h). To determine cysteine and methionine the preparations underwent preliminary oxidation by performic acid. Amino acid composition was calculated on the basis of molecular masses of 12000 for ferredoxin I and 18000 for ferredoxin II. Values are averaged from three measurements for each ferredoxin

Amino acid	Ferredoxin I	Ferredoxin II
Lysine	5	8
Histidine	2	5
Arginine	0	4
Tryptophan	0	2
Aspartate + asparagine	18	21
Threonine	10	12
Serine	3	7
Glutamate + glutamine	13	27
Proline	10	8
Glycine	6	10
Alanine	13	14
Cysteine	9	4
Valine	5	13
Methionine	2	1
Isoleucine	6	11
Leucine	4	8
Tyrosine	1	0
Phenylalanine	0	7
Total amount of residues	107	162

nal amino acid testifies to the presence of a single polypeptide chain or chains containing identical N-terminal amino acids in *Rps. capsulata* ferredoxins.

Redox potential

Redox potentials of ferredoxins measured by potentiometric titration with dithionite at pH 7.5 were shown to be −270 mV for ferredoxin I, and −419 mV for ferredoxin II.

EPR spectra

Rps. capsulata ferredoxin I had spectra both in the oxidized and reduced states (Fig. 2). The signal amplitude of reduced ferredoxins was higher than that of the oxidized ones. A signal with a g value of 2.013 was observed in the EPR spectrum of oxidized ferredoxin I. After reduction with dithionite it changed for g values of 1.90, 1.96 and 2.02. Such EPR spectra are also characteristic of ferredoxins of clostridial type 2 [4Fe-4S] acting as two-electron carriers.

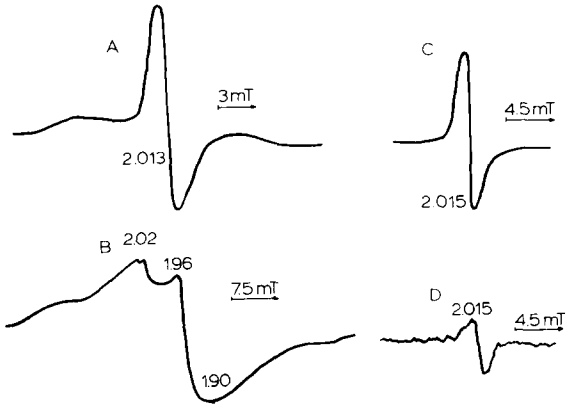

Fig. 2. EPR spectra of oxidized and dithionite-reduced ferredoxins from *Rps. capsulata*. Oxidized ferredoxins I (A) and ferredoxins II (C). Reduced ferredoxins I (B) and ferredoxins II (D). EPR conditions: microwave power, 0.5 mW; modulation amplitude, 7.5 G for A and B, 3.6 G for C and D; temperature, 11 K for A and B, 17 K for C and D. Gain of the measurements for B is 4-times less than that for A, and for C 4-times less than that for D.

In the EPR spectrum ferredoxin II a signal with $g = 2.015$ was observed for the oxidized state (Fig. 2). Reduction by sodium dithionite caused the EPR signal of ferredoxin II to disappear. This may indicate the presence of a [3Fe-3S] cluster in ferredoxin II as in the case of *D. gigas* [30] of ferredoxin II or ferredoxin from *Methanosarcina barkeri* [31]. To elucidate the structure of cluster it seems necessary to make additional EPR studies and experiments on cluster extrusion for both ferredoxins of *Rps. capsulata*.

Stability

Ferredoxins from *Rps. capsulata* differ greatly in their stability. Ferredoxin I was more labile to inactivation by O_2 (Table IV). Its inactivation half-life ($T_{1/2}$) under air (30°C) was about 30 min, while under the same conditions no significant changes in ferredoxin II activity were observed during 2 h.

A decrease in absorption at 380 nm was found when ferredoxin I was incubated under air which was apparently due to the destructive effect of oxygen on Fe-S clusters. The period of 'optical'

TABLE IV

STABILITY OF *RPS. CAPSULATA* FERREDOXIN I UNDER VARIOUS CONDITIONS OF INCUBATION

Ferredoxin I preparations (1.6 mg protein/ml) were incubated under the given conditions for 30 min at 30°C. At the initial stage and after incubation the activity of samples (50 μl) was estimated (see Materials and Methods). Ferredoxin I activity 30 min after incubation is expressed in per cent of ferredoxin I activity at the initial moment of its incubation with inhibitors. SOD, superoxide dismutase.

Argon	Activity (%)	Air	Activity (%)
Without additions	100	Without additions	50
H_2O_2 (5 mM)	20	SOD	45
H_2O_2 + SOD (1 mg)	22	catalase	46
H_2O_2 + catalase (0.6 mg)	90	SOD + catalase	74
H_2O_2 + SOD + catalase	88	sodium ascorbate (1 mM)	68
O_2^- [a]	30	D,L-methionine (5 mM)	58
O_2^- + SOD	46	D,L-histidine (5 mM)	42
O_2^- + catalase	40	mannitol (100 mM)	58
O_2^- + SOD + catalase	96		
$H_2O_2 + O_2^-$	23		
$H_2O_2 + O_2^-$ + SOD	44		
$H_2O_2 + O_2^-$ + catalase	52		
$H_2O_2 + O_2^-$ + SOD + catalase	91		

[a] 14% KO_2 saturated solution in dimethyl sulphoxide.

half-inactivation of ferredoxin I in air was also about 30 min. This suggests that the integrity of ferredoxin I Fe-S clusters is an essential requirement of its functional activity.

It is not only oxygen but also the products of its reduction, O_2^- and H_2O_2, that are known to exert inactivating effects on nitrogenase from *Rps. capsulata* [32]. Catalase protected effectively ferredoxin I against the inactivating effect of H_2O_2 and superoxide dismutase as well as against that of O_2^- under anaerobic conditions (Table IV). Under the simultaneous action of H_2O_2 and O_2^- on ferredoxin I the most efficient protective effect was shown in the presence of both superoxide dismutase and catalase. The combined action of these enzymes produced a protective effect on ferredoxin I while there was no such effect in case of their separate actions.

A protective effect on ferredoxin I was also exhibited by sodium ascorbate which acted as a scavenger of the active forms of oxygen O_2^-, OH· and $^1O_2^*$ [33] and thus protected ferredoxin I against inactivation by air (Table IV). Unlike sodium ascorbate, such scavengers of oxygen active forms (OH· and $^1O_2^*$) as methionine, histidine and mannitol [34] had only a negligible effect on ferredoxin I stability during its incubation in the air.

Ferredoxin I was also shown to have low resistance to heating. After 60 min incubation under anaerobic conditions (+50°C) a 50% decrease in activity took place while unchanged activity of ferredoxin II under the same conditions was observed for 5 h. Both forms of ferredoxin were, however, quickly inactivated at 70°C ($T_{1/2} = 5$ min). This indicates a similar mechanism of their inactivation at this temperature. During the long-term storage (2–3 weeks) under anaerobic conditions at 8°C ferredoxin I was not stable either. Under these conditions the greater part of its molecules was polymerized. This polymeric ferredoxin was eluted in gel filtration with Sephadex G-75 at the void volume. Unlike ferredoxin I storage of ferredoxin II under the same conditions did not result in polymerization of its molecules.

Role in nitrogen fixation and hydrogen metabolism

Both ferredoxins from *Rps. capsulata* were shown to be reduced in a system with pea chloroplasts and they were able to act as electron donors for nitrogenase of this bacterium according to its activity measurements by the reactions of C_2H_2 reduction, or H_2 evolution (Table II and Fig. 3).

In the presence of both ferredoxin I and ferredoxin II, nitrogenase activity estimated from the H_2-evolution assay was considerably higher than that from the C_2H_2-reduction test. This was possibly due to the fact that during acetylene reduction nitrogenase simultaneously catalyzed the reaction of H_2 evolution which required a certain number of electrons from reduced ferredoxin. The rate of H_2 evolution and C_2H_2 reduction in the presence of ferredoxin I was twice as high as that in the case of ferredoxin II addition (Tables II and V). In the presence of both ferredoxins the nitrogenase activity was not higher than that observed when they were added separately to the reaction mixture.

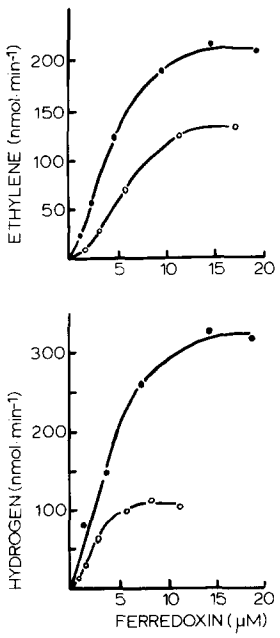

Fig. 3. Dependence of *Rps. capsulata* nitrogenase activity on ferredoxin concentration in the system with pea chloroplasts. For measuring conditions see Materials and Methods. (●——●) Ferredoxin I, (○——○) ferredoxin II.

TABLE V

ACTIVITY OF NITROGENASE OF *RPS. CAPSULATA* IN THE PRESENCE OF VARIOUS ELECTRON DONORS

Nitrogenase activity was determined as reported in Materials and Methods. When $Na_2S_2O_4$ was used as electron donor, ascorbate, DCIP and chloroplasts were eliminated from the reaction mixture

Electron donor	Nitrogenase activity (nmol C_2H_4 reduced/ min per mg protein)
$Na_2S_2O_4$ (10 mM) [a]	34
Rps. capsulata ferredoxins	
ferredoxin I (5 μM)	122
ferredoxin II (5 μM)	60
ferredoxin I (2.5 μM)	
+ ferredoxin II (2.5 μM)	98
ferredoxin I (5 μM)	
+ ferredoxin II (5 μM)	190
Azotobacter vinelandii	
ferredoxin (6 μM)	38
Spirulina platensis	
ferredoxin (15 μM)	49
Pea ferredoxin (21 μM)	30
Spinach ferredoxin (30 μM)	21
Ankistrodesmus brauni	
flavodoxin (80 μM)	8

[a] In the dark, all other variants in the light.

Nitrogenase activity was shown to be higher in the presence of ferredoxins of *Rps. capsulata* than with ferredoxins from other organisms. When dithionite was used as a donor of electrons the nitrogenase activity was about 25–58% of that observed with ferredoxins of *Rps. capsulata* (Table V).

Both forms of ferredoxin from *Rps. capsulata* were not capable of acting as donors or acceptors of electrons for hydrogenases of this bacterium and the purple sulphur bacterium *Thiocapsa roseopersicina.*

Regulation of ferredoxin synthesis

The ferredoxin content of *Rps. capsulata* in the partially purified preparations from cells grown under various conditions was estimated on the basis of its ability to act as an electron carrier from the illuminated chloroplasts to nitrogenase of this bacterium (Table VI). According to the experimental results during chloroplast nitrogenase electron transfer *Rps. capsulata* ferredoxins exhibited various activities depending on the growth conditions. This was possibly due to differences in the type and concentration of ferredoxins in the cells.

The lowest ferredoxin content was detected in the preparations of cells grown under anaerobic conditions with excess ammonium in light or under micro-aerobic conditions in the dark (Table VI). Cells grown with ammonium limitation had a higher content of ferredoxin than those grown in the presence of excess ammonium under the same conditions. Preparations from cells grown in light with excess ammonium and lactate limitation in the presence of CO_2 exhibited the highest activity in the chloroplast nitrogenase system. The same is true for preparations from cells grown in the dark under anaerobic conditions on the medium with glutamate.

The presence of ferredoxins from *Rps. capsulata* cells grown under various conditions was determined with disc gel electrophoresis in 15% polyacrylamide gel. The characteristic brown colour of ferredoxin bands made them easily detectable (Fig.

TABLE VI

FERREDOXIN CONTENT IN PARTIALLY PURIFIED PREPARATIONS OF *RPS. CAPSULATA* CELLS GROWN UNDER VARIOUS CONDITIONS

For details see Materials and Methods. Ferredoxin forms and their content were estimated by ferredoxin activity as electron carrier to nitrogenase and by ferredoxin with various R_f values in polyacrylamide gel electrophoresis (see Fig. 4). 1 relative unit is defined as 1 nmol C_2H_4 formed/min per mg protein of ferredoxin preparation isolated from 50 g of cells. n.d., not determined.

Growth conditions of cells	Ferredoxin content (relative units)	
	Excess NH_4^+	NH_4^+ limitation
Light, anaerobic	4 (ferredoxin II)	196 (ferredoxin I + ferredoxin II)
Dark, microaerobic	20 (ferredoxin II)	160 (ferredoxin II)
Dark, anaerobic (glucose + dimethyl sulphoxide)	70 (ferredoxin II)	380 (ferredoxin II) [a]
Light, anaerobic, lactate limitation	24 (ferredoxin II)	n.d.
Light, anaerobic, lactate limitation + CO_2	390 (ferredoxin II)	n.d.

[a] In this case glutamate was used as nitrogen source.

4). Ferredoxin I had higher electrophoretic mobility than ferredoxin II and was well separated during the electrophoresis of both the mixture of homogeneous ferredoxins and the partially purified preparation from nitrogen-fixing phototrophic cells. The partially purified preparations of ferredoxins from cells grown under other conditions were shown to have only ferredoxin II (Fig. 4). However, definite evidence of the absence of ferredoxin I in *Rps. capsulata* cells grown under these conditions can be obtained through immunochemical analysis.

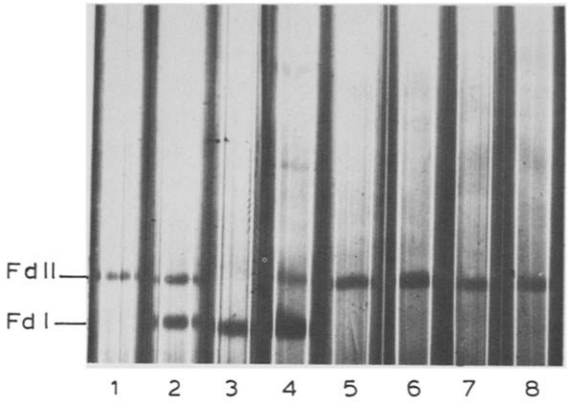

Fig. 4. The nonstained polyacrylamide gels after disc gel electrophoresis of ferredoxin preparations of *Rps. capsulata* cells grown under various conditions. (1–3) Homogeneous preparations of ferredoxin (Fd), (1) ferredoxin II (60 μg), (2) ferredoxin I (80 μg) + ferredoxin II (60 μg), (3) ferredoxin I (80 μg). (4–8) Partially purified preparations of ferredoxin. Growth conditions of cells: (4) light, anaerobic with NH_4^+ limitation (3.4 mg protein); (5) light, anaerobic with excess NH_4^+ (8 mg by protein); (6) anaerobic with excess NH_4^+ in the presence of CO_2 and lactate limitation (0.4 mg protein); (7) dark, microaerobic with excess NH_4^+ (3.2 mg by protein); (8) dark, microaerobic with NH_4^+ limitation (1 mg by protein).

Consequently, ferredoxin I is synthesized by *Rps. capsulata* nitrogen-fixing cells only in light. The nitrogen-fixing cells grown under microaerobic or anaerobic conditions in the dark contain only ferredoxin II, but its content in these cells is considerably higher than in cells not fixing nitrogen. The activity of partially purified ferredoxin preparations from nitrogen-fixing cells grown in the dark similar in the chloroplast nitrogenase system to that of preparations from nitrogen-fixing cells grown in light. A high content of ferredoxin II is also found in the cells grown in light with excess ammonium and lactate limitation in the presence of CO_2 (Table VI). Consequently, the data obtained indicate that the growth conditions affect both the total content and qualitative composition of ferredoxins synthesized in *Rps. capsulata* cells.

Discussion

Hallenbeck et al. [17] isolated and characterized only one ferredoxin of *Rps. capsulata* B10 which is similar in its properties to ferredoxin I described in our paper. Only one ferredoxin has also been isolated from *Rps. capsulata* strain N-3 [15,16]. These authors evidently did not find ferredoxin II

as a result of its low content in nitrogen-fixing phototrophic cells of *Rps. capsulata*.

Similar to the purple non-sulphur bacterium *R. rubrum* [8,35], cells of *Rps. capsulata* are capable of synthesizing two ferredoxins with different properties (Table II). They are similar to *R. rubrum* ferredoxins in the amount of iron and sulphur, spectral characteristics and their functions [13], but differ in the molecular mass and amino acid composition.

The presence of nine cysteine residues in the molecule is also characteristic of *Chromatium vinosum* ferredoxin [36] and of two ferredoxins of *Chlorobium limicola* [37,38]. However, the majority of 8Fe-8S ferredoxins of non-photosynthetic bacteria contain eight cysteine residues [36]. Similar to ferredoxin II of *R. rubrum* [8], ferredoxin II of *Rps. capsulata* contains two tryptophan residues. The N-terminal amino acid, alanine, of ferredoxin I of *Rps. capsulata* is a frequently observed one. The presence of threonine as an N-terminal amino acid was observed for the first time for ferredoxin II of *Rps. capsulata*.

Similar to ferredoxin from the *R. rubrum* carotenoid-less mutant, strain G-9 [39], ferredoxin I of *Rps. capsulata* has a rather high redox potential (Table II).

As with the majority of bacterial ferredoxins [4,11,35], ferredoxin I of *Rps. capsulata* shows an EPR spectrum in both the oxidized and reduced states, whereas ferredoxin II does so only in its oxidized form (Fig. 2). The absorption and EPR spectra, content of non-haem iron and acid-labile sulphur do not provide sufficient information to substantiate final conclusion of the types of clusters in *Rps. capsulata* ferredoxins. Studies of the cluster structure of these ferredoxins are the object of our further research.

Similar to some other bacteria with two ferredoxins [3,35], one of the ferredoxins of *Rps. capsulata* (ferredoxins I) is less stable to the effect of oxygen and heating than the other. The polymerization of ferredoxin I molecules during storage of *Rps. capsulata* preparations with argon is possibly of the same origin as the dimerization of *Cl. pasteurianum* ferredoxin molecules under aerobic conditions [40]. The latter process is due to destruction of one of the ferredoxin Fe-S cluster and formation of intermolecular disulphide bonds. The formation of *Cl. pasteurianum* ferredoxin dimers indicates that the Fe-S clusters in this molecule differ in their stability but the stability of clusters in ferredoxin I of *Rps. capsulata* is apparently the same which results in the formation of a polymeric form of ferredoxin.

Strong inactivation of *Rps. capsulata* ferredoxin I preparations in air during incubation is due to the inactivating effect of O_2 as well as the products of its reduction (Table IV). The presence of superoxide dismutase and catalase in the reaction mixture contributes greatly to the protection of ferredoxin I against O_2 and its active forms (H_2O_2, O_2^-) as in the case of hydrogenase and nitrogenase of this bacterium [32].

Unlike *X. flavus (-M. flavum)* [3] and *R. rubrum* [13], but similar to *Rz. japonicum* [11], ferredoxin I of *Rps. capsulata* is more active as a carrier of electrons from chloroplasts to nitrogenase than dithionite, ferredoxin II of *Rps. capsulata* or ferredoxins of other organisms (Table V). The absence of an acceleration effect in acetylene reduction when both ferredoxins are present in the reaction mixture shows that in vivo the ferredoxins as donors of electrons for nitrogenase function independently of one another.

In case of *X. autotrophicum* [41], a considerable amount of ferredoxin is observed only in its nitrogen-fixed cells, and in case of *R. rubrum* [8] synthesis of ferredoxins I is affected by light. As for *Rps. capsulata*, ferredoxin I synthesis is affected not only by light but also by the nitrogen source in the growth medium (Table VI). This is also possible for *R. rubrum* because two ferredoxins of this bacterium have been observed only in nitrogen-fixing cells grown in light [42] and without a high content of NH_4^+ [43].

So, the presence of light and ammonium-limited growth medium are necessary conditions for ferredoxin I synthesis in purple non-sulphur bacteria under anaerobic conditions. This is proved by the absence of ferredoxin I in the case of *Rps. capsulata* cells grown in light with excess ammonium or in the dark with limited ammonium. It probably means that the function of ferredoxin I is light-dependent electron transport to nitrogenase. The assumption is supported by the following fact: its redox potential (−270 mV) is much closer to the potential of the primary electron acceptor of non-

sulphur purple bacteria (−150 mV) than that of ferredoxin II (−419 mV). Nitrogenase activity of *Rps. capsulata* cells grown in the dark is, however, substantially higher during measurements in light [44]. This fact seems to prove that ferredoxin II may replace ferredoxin I in the system of light-dependent electron transport to nitrogenase. In addition, ferredoxin II can function as an electron donor for nitrogenase during nitrogen-fixing growth in the dark, as under these conditions its level grows dramatically (Table VI).

In addition of nitrogen fixation ferredoxin II seems to participate in other processes, e.g., in fixation of CO_2. This is indicated by the increase in its level which accompanies *Rps. capsulata* growth in light with excess ammonium and limited lactate in the presence of CO_2 (Table VI). In the case of *R. rubrum*, ferredoxin (Fd) (probably ferredoxin II) participates in pyruvate synthesis [45]:

$$\text{Acetyl-CoA} + CO_2 + Fd_{red} \rightarrow \text{pyruvate} + \text{CoA} + Fd_{ox}$$

Practically nothing is known about ferredoxin-dependent reactions occurring during *Rps. capsulata* growth in the dark under anaerobic conditions in the presence of dimethyl sulphoxide and glucose. Oxidative cleavage of pyruvate is assumed to take place under these conditions, yielding acetyl-CoA and CO_2 [46], i.e., the reverse reaction of CO_2 fixation. Reduced ferredoxin II formed in this reaction can be used as an electron donor for nitrogenase. Elucidation of ferredoxin II functions in the cells of *Rps. capsulata* requires further research.

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