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Localization and characterization of Fe(Ⅲ) reductase of *Desulfovibrio dechloracetivorans* strain SF3

WANG Jia-chen, LIN Yang, SUN Bao-lin

(*Hefei National Laboratory for Physical Sciences at Microscale, and School of Life Sciences, University of Science and Technology of China, Hefei* 230027, *China*)

Abstract: Both physiological and biochemical studies have demonstrate that Fe(Ⅲ)-EDTA is a favorite soluble Fe(Ⅲ) of strain SF3. The UV-visible spectra of dithionite-reduced, soluble Fe(Ⅲ) reoxidized cell extracts indicates the involvement of c-type cytochromes in Fe(Ⅲ) reduction. Fractionation experiments suggest that 87% of the Fe(Ⅲ) reducing activity is localized at soluble fraction, 74% of the Fe(Ⅲ)-reducing activity is found in the spheroplasts, which may indicate a distinct mechanism for Fe(Ⅲ) reduction. Fe(Ⅲ) reductase of strain SF3 exhibits the highest activity at 25 ℃, pH 7.4, and shows a great oxygen-tolerance ability.

Key words: Fe(Ⅲ) reductase; *Desulfovibrio dechloracetivorans* strain SF3; localization; characterization

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Desulfovibrio dechloracetivorans strain SF3 三价铁还原酶的定位和性质的研究

王佳晨,林 洋,孙宝林

(中国科学技术大学生命科学学院,合肥微尺度物质科学国家实验室,安徽合肥 230027)

摘要:生理和生化实验结果表明Fe(Ⅲ)-EDTA是SF3偏好的一种可溶性三价铁.连二亚硫酸盐还原后的SF3经过可溶性三价铁氧化后,其紫外可见光谱显示c型细胞色素参与了三价铁的还原过程.铁还原酶的定位实验说明87%的铁还原酶活性定位于细胞可溶性组分,74%的活性分布于原生质体中,这种分布情况可能与一种和其他细菌不同的铁还原机制有关.SF3的铁还原酶在25 ℃, pH 7.4时显示最高活性,并且在氧气中表现出较强的稳定性.

关键词:三价铁还原酶;*Desulfovibrio dechloracetivorans* strain SF3;定位;性质

0 Introduction

Iron is one of the most abundant elements in the Earth. Geochemical and microbiological evidences suggests that dissimilatory Fe(Ⅲ) reduction might be the first form of microbial

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Biography: WANG Jia-chen, female, born in 1983, master. Research field: dissimilately reductase of anaerobic bacteria. E-mail: jcwang@mail.ustc.edu.cn

Corresponding author: SUN Bao-lin, professor. E-mail: sunb@ustc.edu.cn

respiration[1~3]. The abundance of iron in submerged soils and aquatic sediments and the fact that Fe(Ⅱ) tends to be reoxidized at the oxic/anoxic interface due to its solubility and mobility[4] suggest that dissimilatory Fe(Ⅲ) reduction may play a significant role in the geochemistry of these environments. In addition, most Fe(Ⅲ)-reducing microorganisms can degrade various kinds of organic compounds for Fe(Ⅲ) reduction[5~7] and account for 35% ~ 65% of the organic-matter oxidation in anaerobic sediments[5~7]. Thus, dissimilatory Fe(Ⅲ) reducers may have the potential to play an important role in the bioremediation of organic pollutants in many contaminated sediments[4,8].

Fe(Ⅲ) reducing microorganisms are widely distributed in many genera[4~6], and have been intensively investigated in recent two decades. Sulfate reducing bacteria (SRB), a kind of microorganism that can dissimilatively reduce sulfate to sulfide, sulfite or thiosulfate, is an important group of Fe(Ⅲ) reducers. SRBs are widely distributed in many niches and capable of enzymatically reducing many heavy metals including Fe(Ⅲ), thus playing a great role in global iron cycle and in the biocorrasion of man-made ironworks[6,9,10]. Besides these significances, metal reduction by SRB may greatly stimulate the degradation of organic matters and might be a very important inhibitory factor to biotic sulfate reduction in sediments[9,10], thus greatly affecting the sulfur and carbon cycles in many environments. However, though metal reduction by intact cells of SRB has been intensively studied, little investigation was focused on the Fe(Ⅲ)-reducing mechanism of these microorganisms[4,11].

Here we investigate the localization and characteristics of Fe(Ⅲ) reductase of *Desulfovibrio dechloracetivorans* strain SF3, a marine chlorophenol-respiring, sulfate-reducing bacterium[12], which is capable of reducing Fe(Ⅲ) dissimilatively. Results from physiological and biochemical studies indicate that strain SF3 reduces Fe(Ⅲ) in a distinct manner from many other SRBs and has a different distribution of ferric reductase from previously characterized species in *Geobacter* and *Shewanella*[11,13~15].

1 Materials and methods

1.1 Bacterial strain and culture conditions

Standard anaerobic techniques were used in all experiments. *D. dechloracetivorans* strain SF3 (ATCC 700912) was routinely grown in 160 mL or 1 L glass serum bottles with 100 mL or 600 mL of modified seawater media as previous described[12]. No sulfate source is present in the basal medium to avoid chemical reduction of metal ions. Sterilized electron donors and acceptors were added from anaerobic stock solutions.

For bacterial cultivation, 1 mmol/L Na_2S as the reductant, 10 mmol/L sodium pyruvate as the electron donor, 30 mmol/L sodium sulfate as the electron acceptor, plus 0.1% yeast extract were added into 600 mL medium prior to a 1% inoculation from 2.5 mmol/L acetate —250 μmol/L 2-chlorophenol growth culture.

1.2 Cell preparation and metal reduction

To testify the ability of whole cells of strain SF3 to reduce Fe(Ⅲ), cells were harvested at the end of the logarithmic growth phase by centrifugation at 10 000 g for 30 min, washed twice and resuspended in anaerobic bicarbonate buffer ($NaHCO_3$, 30 mmol/L; NaCl, 427 mmol/L; $MgCl_2$, 14 mmol/L; $CaCl_2$, 0.9 mmol/L; pH 7.4). 0.1 mL of the cell suspension was added to 30 mL anaerobic basal medium in a 50 mL serum bottle bubbled with N_2-CO_2 (95 ∶ 5) to a final density of ca. 3×10^6 cells/mL. Different kinds of soluble Fe(Ⅲ) solution and excess electron donors were added from sterile anaerobic solutions by syringe needles. 10 mmol/L Fe(Ⅲ)- EDTA, Fe(Ⅲ)-NTA or ferric citrate was added as the electron acceptor when formate at 10 mmol/L or H_2 at 44 mmol/L was evaluated as an electron donor for Fe(Ⅲ) reduction.

1.3 Preparation of subcellular fractions

All enzymatic experiments were conducted in an anaerobic glove box. Late logarithmic phase grown cells were harvested as described above. Cells were washed twice, and then resuspended with N_2-saturated phosphate buffer (50 mmol/L, pH7.0). To obtain the membrane and soluble fraction, a modified method was used[16]: cells were broken through a French pressure cell for three times. Unbroken cells and debris were removed by centrifugation at 10 000 g for 15 min. Membranes and soluble fractions were separated by ultracentrifugation (100 000 g, 4 ℃, 2 h). Separation of spheroplasts and periplasmic fraction was operated according to the protocol as previous described[13]. Cells were resuspended in anaerobic phosphate buffer containing 25% (wt/vol) sucrose. Lysozyme (final concentration of 0.64 $g \cdot L^{-1}$) and Na_2-EDTA (final concentration of 2 $g \cdot L^{-1}$) were added from freshly prepared anaerobic solutions drop by drop with constant stirring at 15 min intervals. Then 0.1 mol/L $MgCl_2$ solution was added to a final concentration of 12.8 mmol/L. After that, spheroplasts and periplasmic fractions were separated by centrifugation at 20 000 g for 30 min. Spheroplasts (pellet) were resuspended in phosphate buffer. After preparation, all fractions were used as soon as possible to avoid loss of metal reduction activity.

1.4 Enzyme assay

Fe(Ⅲ) reductase activities were assayed anaerobically by spectrophotometric method. Appearance of Fe(Ⅱ) was monitored over time colorimetrically (see section 1.6). The reaction mixture for metal reduction contained 1 mmol/L NADH as electron donor and either 0.5 mmol/L Fe(Ⅲ)-EDTA, Fe(Ⅲ)-NTA, or ferric citrate as electron acceptor in anaerobic phosphate buffer. The enzyme assay was started by adding cell extracts or different fractions (0.05～3 $g \cdot L^{-1}$ of protein) to both sample and reference test tube. Reaction mixtures were incubated at 30 ℃ in the dark. Fe(Ⅱ) concentration of HCl ferrozine treated sample was determined by measuring the A_{562} against a reference that had no NADH. Fe(Ⅲ)-NTA was synthesized using Roden and Lovley's method[17].

The optimal pH for Fe(Ⅲ) reduction by cell extracts of SF3 were evaluated using anaerobic phosphate buffers adjusted to pH 5.99, 6.49, 6.78, 7.01, 7.21, 7.40, 7.59 and 7.96. pH values were certified before and after the experiment to make sure that no changes in pH occurred in the period of reaction. The optimal temperature determination was conducted at 4, 16, 20, 25, 28, 30, 33, 37, 45, 50 and 60 ℃.

To test temperature stability of Fe(Ⅲ) reduction activity, cell extracts of SF3 were resuspended in anaerobic phosphate buffer and incubated anaerobically in the dark at 4, 20, 30, 35, 40, 45, 50, 60, 70, and 90 ℃ for 1 hour. Then a 1/10 volume of cell extracts in each temperature was added into the reaction mixture for Fe(Ⅲ) reduction as described above and incubated at 30 ℃ to measure the remanent Fe(Ⅲ) reduction activity. For oxygen stability experiments, cell extracts were resuspended in aerobic phosphate buffer and exposed to air in the dark at 30 ℃. Samples were collected over time and Fe(Ⅲ) reduction activity was measured.

1.5 UV-visible spectroscopy

UV-visible spectra of cell extracts of strain SF3 were recorded on a spectrophotometer in a 3 mL glass cuvette containing freshly prepared cell extracts. The protein solution was firstly reduced with 250 μmol/L sodium dithionite and subsequently reoxidized by 125 μmol/L Fe(Ⅲ)-EDTA, Fe(Ⅲ)-NTA or ferric citrate, respectively.

1.6 Chemical assay and data analysis

Fe(Ⅱ) concentration was monitored spectrophotometrically by a modified acid extraction-ferrozine method[18] at 562 nm. Protein concentration was measured using Bradford method[19]. Syringe needles were used in all sampling processes and measurements of metal ions were conducted in an anaerobic glove box.

All results were the mean values of three

independent measurements, standard deviations are shown in all table and figures.

2 Results and discussion

2.1 Fe(Ⅲ) reduction by strain SF3

For Fe(Ⅲ) reduction is one of the most notable properties in most of the SRBs[6,9,10]. It's not surprising that strain SF3 could reduce Fe(Ⅲ)-EDTA in the presence of H_2 or formate in our experiment: the brown color of 10 mmol/L Fe(Ⅲ)-EDTA disappeared after several days of incubation, and the concentration of Fe(Ⅱ) changed from 0 mmol/L to 10 mmol/L. Fe(Ⅲ)-NTA could also be reduced when H_2 or formate served as the electron donor, though the reduction rate was several times lower than that of Fe(Ⅲ)-EDTA (data not shown). However, ferric-citrate, which was widely used as the source of chelated Fe(Ⅲ) in many former metal reduction experiments[13], could be totally reduced without extra electron donors added (data not shown). One possible explanation for this phenomenon is that citrate, the chelate agent, can be used as an electron donor by strain SF3 for Fe(Ⅲ) reduction.

2.2 Localization of Fe(Ⅲ) reductase

Localization of Fe(Ⅲ) reductase was evaluated when NADH served as electron donor and Fe(Ⅲ)-EDTA as electron acceptor. Subcellular fractions of strain SF3 including cell extracts, membrane fractions, soluble fraction, periplasm and spheroplasts did not reduce Fe(Ⅲ) without the addition of NADH. In the presence of NADH, breakage of the cells led to a 23% increase in ferric iron reductase activity compared with that of whole cells (Tab. 1). The soluble fractions of crude extracts accounted for about 87% of ferric iron reductase activity. The periplasm contained 26% of ferric iron reductase activity (Tab. 1). This distribution is not consistent with those former experiments[11,13~15] and might be responsible for a different mechanism of Fe(Ⅲ) reduction by strain SF3, which will be discussed below.

2.3 Influence of different chelate agents on Fe(Ⅲ) reduction by Fe(Ⅲ) reductase of strain SF3

The ferric iron reductase activity of strain SF3 was measured when NADH was used as electron donor. In the absence of electron donor, cell extract of strain SF3 did not reduce Fe(Ⅲ)-EDTA, Fe(Ⅲ)-NTA or ferric citrate. When NADH exists, in the three forms of chelated Fe(Ⅲ), Fe(Ⅲ)-EDTA was most favourable (18.6 mU/mg of protein, an activity of 1 mU corresponds to 1 nmol of metal formed per min) and lower activity was measured with Fe(Ⅲ)-NTA (3.1 mU/mg of protein) and ferric citrate (1.9 mU/mg of protein). The ferric iron reductase activity with Fe(Ⅲ)- EDTA as electron acceptor was 6 folds

Tab. 1 Localization of Fe(Ⅲ), Cr(Ⅵ) and Co(Ⅲ) reductase in different cellular fractions of strain SF3

fraction	protein/mg	Fe(Ⅲ) reductase activity[a]		
		Sp act/(mU · mg^{-1})	total activity/mU	%
whole cells vs. crude extract				
whole cells	11.9±0.5	5.3±0.2	62.8±2.2	—
crude extract	14.5±0.1	6.5±0.2	94.2±2.8	—
soluble fraction vs. membrane fraction				
membrane fraction	2.7±0.3	4.9±0.1	13.1±0.3	13
soluble fraction	5.9±0.1	14.7±2.3	86.6±13.4	87
periplasm vs. spheroplasts				
periplasm	4.4±0.4	1.8±0.3	7.8±1.2	26
spheroplasts	4±0.1	5.6±0.9	22.4±3.5	74

【Note】 [a]Reaction mixture contained 1 mmol/L NADH and 0.5 mmol/L Fe(Ⅲ)-EDTA, K_2CrO_4 or Co(Ⅲ)-EDTA respectively in 50 mmol/L anaerobic phosphate buffer (pH7.0). An activity of 1 mU corresponds to 1 nmol of each metal formed per min. Results were the mean values of three independent measurements, and standard deviations are shown after the mean values.

higher than that with Fe(Ⅲ)-NTA and about 10 folds higher than with ferric citrate.

The preference of EDTA by Fe(Ⅲ) reductase of strain SF3 was unexpected because in former investigations, EDTA chelated Fe(Ⅲ) could not be readily reduced by Fe(Ⅲ) reducing microorganisms[13,20,21]. In addition, in the ferric iron reductase study on *Geobacter sulfurreducens*[13] and the whole cells investigation on *Shewanella putrefaciens*[20], Fe(Ⅲ)-NTA and ferric-citrate, respectively, induced the highest Fe(Ⅲ) reducing activity. Haas et al.[20] suggested that this phenomenon was attributed to the difference of thermodynamic stability of Fe(Ⅲ) chelates, and the Fe(Ⅲ) reducing activity was restrained by the competition for Fe(Ⅲ) between organic chelates and the outer bacterial surface. However, in our experiments, intact cells of strain SF3 reduced Fe(Ⅲ)-EDTA faster than Fe(Ⅲ)-NTA, which was also observed in cell extracts experiment. And ferric-citrate was reduced at the lowest rate by Fe(Ⅲ) reductase of strain SF3. We proposed that the different partialities for soluble Fe(Ⅲ) chelates is due to a different mechanism of Fe(Ⅲ) reduction by strain SF3. In localization experiments on *Geobacter* and *Shewanella*[11,13~15], the ferric iron reductase activity was mostly associated with the outer membrane fraction. Moreover, ferric iron reductase activity of *G. sulfurreducens* could be washed from intact cells by 0.5 mol/L KCl, indicating that the ferric iron reductase of *G. sulfurreducens* is peripheral to the outer membrane[13]. Apparently, this outer-membrane localization of ferric iron reductase is favorable for the utilization of solid ferric irons. However, in our study, 87% of the ferric iron reductase was located at the soluble fraction (Tab. 1). Considering that 74% of the activity was distributed into the spheroplasts, a cytoplast-localization of ferric iron reductase of strain SF3 was suggested. This distribution indicates that a different Fe(Ⅲ) reducing mechanism is employed by strain SF3 and probably some other non-growth Fe(Ⅲ) reducers. For that reason, competition for Fe(Ⅲ) ions between organic chelates and the outer bacterial surface may no longer be a rate-limiting step, making the thermodynamic stability of Fe(Ⅲ) chelates no more a crucial property for Fe(Ⅲ) reduction.

2.4 Differences between Fe(Ⅲ) reduction by intact cells or cell extracts of strain SF3

Some characteristics of Fe(Ⅲ) reduction by strain SF3 were changed in the subcellular fractions preparation process. The probable reason was the disturbance of the respiratory chain. For example, the ability to directly reduce ferric-citrate without the addition of any other electron donor was lost in subcellular fractions. Another difference between intact cells and cell extracts of strain SF3 was the increase of Fe(Ⅲ) reduction activity after the breakage of intact cells (Tab. 1). Similar results have been obtained in many experiments on metal reduction of other microorganisms[22].

2.5 Optimal pH and temperature for Fe(Ⅲ) reductase of strain SF3

The optimal pH for ferric iron reductase activity of strain SF3 was 7.4 (Fig. 1(b)), which was also the optimal pH for reductive dechlorination of strain SF3[12]. The highest activity of ferric iron reductase was measured at 25 ℃ (Fig. 1(a)) and a relatively high activity appeared at 25～33 ℃, which is also compliant with the optimal temperature for dechlorination.

2.6 Oxygen and temperature stability

A great feature of ferric iron reductase of strain SF3 is its oxygen-tolerance (Fig. 2(b)). Ferric iron reductase activity of strain SF3 decreased fast in the first 6 hours when exposed to air and approximately 50% activity was lost after 6 hours incubation in air, and the activity decreased steadily at a lower speed (Fig. 2(b)). Trace (0.78 mU/mg protein) activity could still be measured after 50 hours exposure to air. In contrast, *G. sulfurreducens* lost 80% of its ferric iron reductase activity after incubation in air for 2 h[13], and even

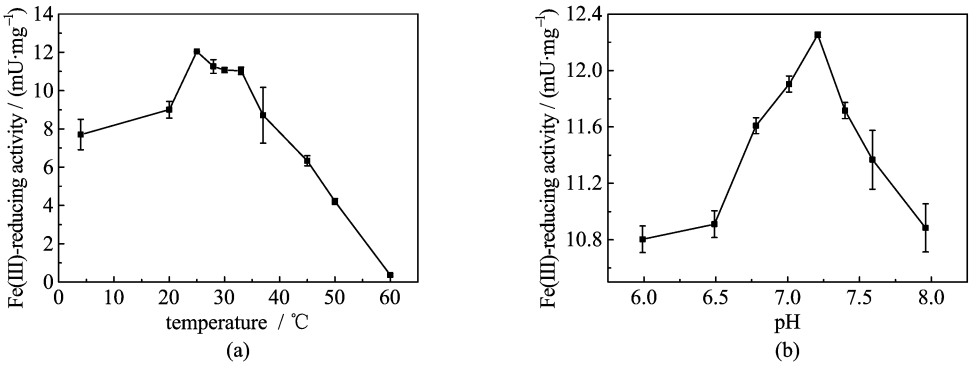


Fig. 1 Optimal conditions for ferric iron reductase of strain SF3

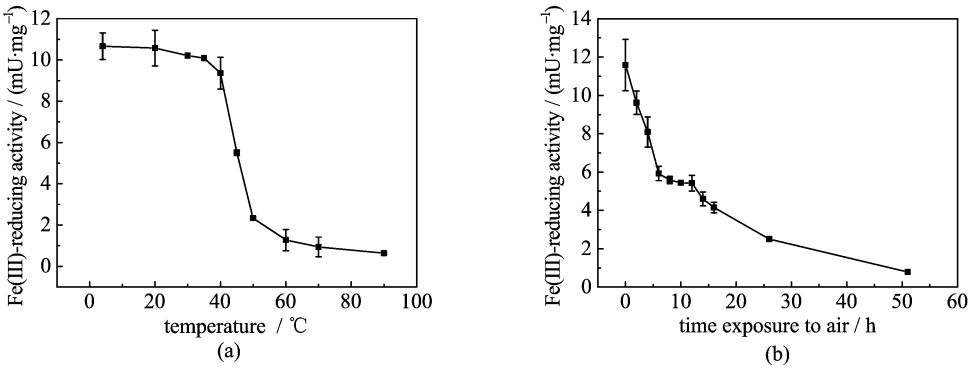


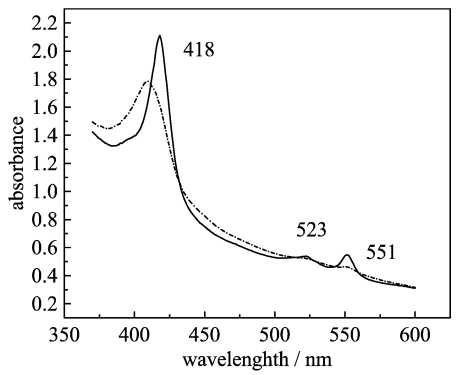
Fig. 2 Temperature and oxygen sensitivity of ferric iron reductase activity

5 min exposure to air would irreversibly inhibit Fe(Ⅲ) reductase activity of strain GS-15[23].

For temperature stability, cell extracts of strain SF3 incubated at 4 ～ 40 ℃ for 1 hour retained more than 85% of the ferric iron reductase activity (Fig. 2(a)). Approximately 50% or 80% activity was lost when the incubation temperature was 45 ℃ or 50 ℃, and less than 12% activity was retained when the incubation temperature was higher than 60 ℃.

2.7 UV-visible spectroscopy of Fe(Ⅲ) reductase

The UV-visible spectra of dithionite-reduced cell extracts exhibited a typical absorbance curve of c-type cytochromes. The strongest peak was observed at 418 nm and another two peaks appeared at 523 and 551 nm. The addition of Fe(Ⅲ)-EDTA, Fe(Ⅲ)-NTA or ferric citrate into the cuvette led to a shrinkage of these peaks, indicating that these c-type cytochromes are involved in Fe(Ⅲ) reduction (Fig. 3), a result compliant with the hypothesis that c-type cytochrome plays an important role in bacterial metal reduction[4,11,24,25].



solid line—UV-visible spectra of cell extracts reduced with dithionite; dashed line—spectra of Fe(Ⅲ)-EDTA reoxidized cell extracts

Fig. 3 UV-visible spectra experiment of cell extracts of strain SF3

3 Conclusion

Though c-type cytochrome is also involved in the Fe(Ⅲ) reduction, the Fe(Ⅲ) reductase of strain SF3 is mostly located in the cytoplast, which is a different distribution from many other investigated microorganisms. This distribution, possibly, is an implication of a different strategy for Fe(Ⅲ) reduction by strain SF3 than bacteria in

Geobacter and *Shewanella*, which might be responsible for the preference of Fe(Ⅲ)-EDTA. Many other unique features, such as the oxygen-tolerance and different performances before and after the cell breakage, also suggest that the mechanism of Fe(Ⅲ) reduction by strain SF3, or even many other SRBs, is different from those former investigated bacteria.

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