Acta Geochim (2017) 36(3):544–547
DOI 10.1007/s11631-017-0232-9

ORIGINAL ARTICLE

The in situ spectral methods for examining redox status of *c*-type cytochromes in metal-reducing/oxidizing bacteria

Xiaobo Luo[1,2,3] · Yundang Wu[2] · Xiaomin Li[2] · Dandan Chen[2] · Ying Wang[2] · Fangbai Li[2] · Tongxu Liu[2]

Received: 12 March 2017 / Revised: 22 May 2017 / Accepted: 16 August 2017 / Published online: 24 August 2017

Abstract The membrane-associated *c*-type cytochromes (*c*-Cyts) have been well known as the key enzymes mediating extracellular electron transfer to terminal electron acceptors, resulting in biogeochemical elemental transformation, contaminant degradation, and nutrient cycling. Although *c*-Cyts-mediated metal reduction or oxidation have been mainly investigated with the purified proteins of metal reducing/oxidizing bacteria, the in vivo behavior of *c*-Cyts is still unclear, given the difficulty in measuring the proteins of intact cells. Fortunately, the in situ spectroscopy would be ideal for measuring the reaction kinetics of *c*-Cyts in intact cells under noninvasive physiological conditions. It can also help the establishment of kinetic/thermodynamic models of extracellular electron transfer processes, which are essential to understand the electron transfer mechanisms at the molecular scale. This review briefly summarizes the current advances in spectral methods for examining the *c*-Cyts in intact cells of dissimilatory metal reducing bacteria and Fe(II)-oxidizing bacteria.

Keywords *c*-type cytochrome · In situ spectroscopy · Intact cells · Metal reducing bacteria · Fe(II) oxidation bacteria

1 Introduction

The electron transfer processes between microorganisms and minerals affects a series of environmental processes, such as pollution degradation and mineral formation. These microorganisms have developed a sophisticated electron transport network. For metal reducing bacteria (e.g. *Shewanella oneidensis* MR-1), the electron transfer chain occurred from the inner to outer membrane (OM) via a metal reduction (Mtr) pathway (Breuer et al. 2014). For iron oxidizing bacteria (e.g. *Sideroxydans lithotrophicus* ES-1), a reverse pathway for metal oxidation (Mto) by transferring electrons from the outer to the inner membrane was observed (Liu et al. 2012). In the electron transfer chains, the *c*-type cytochromes (*c*-Cyts) acted as the active centers mediating electron transfer processes. In the microbe-mineral interfaces, the *c*-Cyts can mediate electron transfers from the cell surface to the terminal electron acceptors (i.e. organics, electrodes, and metals), or directly acquire electrons from the electron donors [i.e. Fe(II)], resulting in metal reduction/oxidation. Thus *c*-Cyts is an important component of the biogeochemical cycling of metals and microbial activities in anoxic subsurface environments (Shi et al. 2016). Therefore, the *c*-Cyts have attracted great attention with regards to the biogeochemical processes in microbe-mineral interfaces. The traditional approach of investigating the property of key electron transfer proteins is to extract and purify these proteins from the cells. For example, the key electron transfer proteins of *Shewanella oneidensis* MR-1, OmcA and MtrC were

11th International Symposium on Geochemistry of the Earth's Surface.

✉ Tongxu Liu
txliu@soil.gd.cn

[1] Guangzhou Institute of Geochemistry, Chinese Academy of Sciences, Guangzhou 510640, People's Republic of China

[2] Guangdong Key Laboratory of Integrated Agro-Environmental Pollution Control and Management, Guangdong Institute of Eco-Environmental and Soil Sciences, Guangzhou 510650, People's Republic of China

[3] University of Chinese Academy of Sciences, Beijing 100049, People's Republic of China

extracted and purified, and then their functional activity was comprehensively characterized (Shi et al. 2016). However, the in vitro behavior of *c*-Cyts was very different from the in vivo behavior, because the proteins interact directly with other proteins along the electron transfer chain, and the highly reactive enzymes may readily be changed during the purification processes (Ross et al. 2009). Hence, the in vivo study of the reaction between minerals and *c*-Cyts in live cells can be valuable for comprehensively understanding the microbial metal redox process. The active center of *c*-Cyts, heme iron, has a large molar absorption coefficient (Nakamura et al. 2009), so the spectral method has been used to characterize the properties of the *c*-Cyts. Due to the nature of UV/Vis spectral methods, only the *c*-Cyts located on the very surface of the cell outer-membrane can be measured directly, while the other *c*-Cyts in the cells may not be detected, and thus this method is suitable for examining the redox status of OM *c*-Cyts in intact cells. Because the absorption peak of the reduced *c*-Cyts is different from the oxidized forms in UV/Vis absorption spectroscopy (Fig. 1a), the concentration of the reduced or oxidized *c*-Cyts can be continuously measured in the reaction solution. Although the iron-reducing bacteria have significant amounts of membrane-associated *c*-Cyts, the application of the UV/Vis absorption spectroscopy to a living microbe has been limited by the strong spectral interference from the light scattering of cell surfaces (Nakamura et al. 2009). Fortunately, by using a diffuse-transmission (DT) mode (Fig. 1b), such interference was not observed in the absorption spectra of *c*-Cyts in whole cells, because an integrated sphere can collect this scattering light of cell surfaces. Therefore, the reaction kinetics between metals and *c*-Cyts can be monitored and calculated. Given the accuracy and rapid measurements without any interruption on intact cells, the spectral method has been increasingly applied for *c*-Cyts characterization recently. This brief review will focus on the recent advances of the in situ spectral methods for *c*-Cyts in the intact cells of iron-reducing/oxidizing bacteria.

2 In situ spectral methods for *c*-Cyts of iron-reducing bacteria

Using DT UV/Vis spectroscopy, the *c*-Cyts in living cell suspension of *Shewanella putrefaciens* 200, were successfully quantified due to the strong absorbance of reduced *c*-Cyts (*c*-Cyt_{red}) at 552 nm, and the in situ spectral kinetics of *c*-Cyts were comprehensively examined, in which the *c*-Cyt_{red} was initially oxidized to an oxidized form (*c*-Cyt_{ox}) by the electron acceptor and then recovered slowly as more and more electron acceptors were reduced over time (Liu et al. 2016). Meanwhile, because the ratio of *c*-Cyt_{ox} and *c*-Cyt_{red} in *Shewanella oneidensis* MR-1 can be obtained simultaneously from the DT UV/Vis spectra, the thermodynamic model can be established on a basis of the Nernst equation, which is useful for disclosing the energetic constrains for extracellular electron transfer in the living cells (Wu et al. 2014). It was found that the oxidation of *c*-Cyt_{red} by Cr(VI) occurred too rapidly (<1 min) to be recorded by a traditional spectrophotometer (Liu et al. 2016). Therefore, an optical-fibre spectrometer was equipped with an integrated-sphere detector to directly examine the rapid reaction kinetics of *c*-Cyts in living cells. The rapid spectral changes during the reaction between 5-hydroxy-1,4-naphthoquinone and *c*-Cyts in the living *Shewanella oneidensis* MR-1 were collected on a second and even 10 ms scale (Liu et al. 2017). Based on the in vivo kinetics and thermodynamics analysis, this study can provide a direct molecular level observation of the in vivo rapid electron transfer processes between *c*-Cyts and electron acceptors.

Despite the above-mentioned progress in the in situ spectral research, there are some limitations to examining *c*-Cyts in intact cells by DT-UV/Vis spectroscopy. First of all, the absorption coefficient of *c*-Cyt_{red} at 552 nm is so small that the quantitative method of in vivo OM *c*-Cyts based on changes in *c*-Cyt_{red} has large errors in the dilute cell suspension. In addition, the OM *c*-Cyts include a series of different kinds of proteins that all contain heme iron as the active center, such as MtrC and OmcA in MR-1, but it

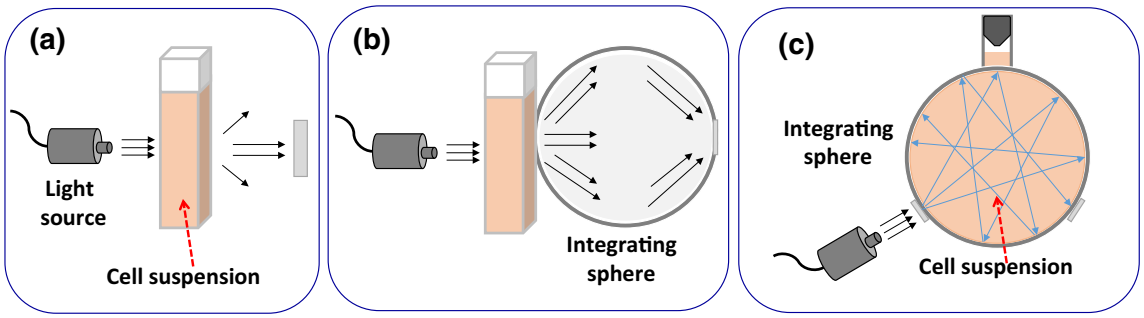

Fig. 1 The schematic diagram of spectral techniques, **a** ordinary absorption mode, **b** diffuse-transmittance absorption mode, and **c** diffuse-transmittance mode with a built-in sample container

is difficult to differentiate the roles of MtrC and OmcA from those of other *c*-Cyts by just using the DT UV/Vis spectral method.

3 In situ spectral methods for *c*-Cyts of iron-oxidizing bacteria

The research on iron-oxidizing bacteria started relatively late as compared with the research on iron-reducing bacteria, so the biological mechanisms and functional proteins were still not well known (Ilbert and Bonnefoy 2013). It was reported that the *c*-Cyts of iron-oxidizing bacteria were also involved in the electron transfer between Fe(II) and cell membranes. Hence, the characterization of *c*-Cyts and their roles in Fe(II) oxidation will be essential for understanding the contributions of biological processes to microbial Fe(II) oxidation. However, due to the lower contents of *c*-Cyts than that of iron-reducing bacteria, it was still hard to accurately measure *c*-Cyts by using the ordinary DT UV/V is spectroscopy. Fortunately, based on the DT mode, a new spectrophotometer with lower detection limits (Fig. 1c) was recently developed to investigate the reaction between ferrous and *c*-Cyts in intact Fe(II)-oxidizing bacteria (*Leptospirillum ferrooxidans*) under anoxic conditions (Blake and Griff 2012). Compared with the ordinary DT UV/Vis spectroscopy, the new sample container is a spherical quartz cuvette and fused with a quartz tube, and the quartz chamber is surrounded by tightly packed proprietary white powder that served to maximize the diffuse reflectance of light on the exterior walls of the spherical flask. Using this spectrophotometer, the in situ spectral kinetics of *c*-Cyts (cytochrome 579) in iron-oxidizing bacteria was successfully examined (Blake and Griff 2012). For microbially-mediated nitrate-reducing Fe(II) oxidation, the nitrite and NO, as the intermediate products of biological nitrate reduction, are able to chemically react with Fe(II) in this system, so the contributions of the biological processes and chemical processes to Fe(II) oxidation might be still unclear. While the underlying mechanisms of key proteins mediating electron transfer have not been well characterized, the in situ spectroscopy is a very promising tool for disclosing the enzymatic mechanisms given the nature of this method for directly observing the key proteins (*c*-Cyts).

4 Perspectives

The previous studies of in situ spectral methods for *c*-Cyts in iron reducing/oxidizing bacteria have obtained some impressive progress from a molecular level under non-invasive physiological conditions. However, there are still some limitations to examining in vivo *c*-Cyts using the in situ spectroscopy. For *c*-Cyts-containing bacteria, because the absorption coefficient of *c*-Cyts at 552 nm in the DT UV/Vis spectroscopy was very weak while the peak at 410/419 nm was relatively high, the accuracy of quantifying the *c*-Cyts could be substantially enhanced by using the absorption peaks at 410/419 nm instead of the peak at 552 nm, and it will be worthwhile to develop systematical methods for *c*-Cyts measurements by splitting the peaks at 410/419 nm. In addition, employing genetic/protein engineering technology to obtain the knockout strains is expected to effectively differentiate the roles of MtrC and OmcA from other *c*-Cyts detected from the DT UV/Vis spectral method. For iron oxidizing bacteria, despite the progress in the in vitro studies on *c*-Cyts and Fe(II), it would be promising to directly investigate the in vivo reactions between Fe(II)-oxidizing proteins and Fe(II) using the in situ spectral method. For microbially-mediated nitrate-reducing Fe(II) oxidation, the in situ spectroscopy may be used to directly measure reaction kinetics between Fe(II) and Fe(II)-oxidizing *c*-Cyts, which would provide direct evidence on biological Fe(II) oxidation, and eventually distinguish the contributions of biological processes and chemical processes to Fe(II) oxidation.

Acknowledgements This work was funded by the National Natural Science Foundations of China (41522105 and 41571130052), Guangdong Natural Science Funds for Distinguished Young Scholar (2014A030306041) and Special Support Program (2016).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

Blake RC II, Griff MN (2012) In situ spectroscopy on intact *Leptospirillum ferrooxidans* reveals that reduced cytochrome 579 is an obligatory intermediate in the aerobic iron respiratory chain. Front Microbiol 3:136

Breuer M, Rosso KM, Blumberger J, Butt JN (2014) Multi-haem cytochromes in *Shewanella oneidensis* MR-1: structures, functions and opportunities. J R Soc Interface 12:20141117

Ilbert M, Bonnefoy V (2013) Insight into the evolution of the iron oxidation pathways. Biochim Biophys Acta Bioenerg 1827:161–175

Liu J, Wang Z, Belchik SM, Edwards MJ, Liu C, Kennedy DW, Merkley ED, Lipton MS, Butt JN, Richardson DJ (2012) Identification and characterization of MtoA: a decaheme *c*-type cytochrome of the neutrophilic Fe(II)-oxidizing bacterium *Sideroxydans lithotrophicus* ES-1. Front Microbiol 3:37

Liu T, Li X, Li F, Rui H, Wu Y, Xiu Y, Ying W (2016) In situ spectral kinetics of Cr(VI) reduction by *c*-type cytochromes in a suspension of living *Shewanella putrefaciens* 200. Sci Rep 6:29592

Liu T, Wu Y, Li F, Li X, Luo X (2017) Rapid redox processes of *c*-type cytochromes in a living cell suspension of *Shewanella oneidensis* MR-1. ChemistrySelect 2:1008–1012

Nakamura R, Ishii K, Hashimoto K (2009) Electronic absorption spectra and redox properties of *c*-type cytochromes in living microbes. Angew Chem Int Ed 48:1606–1608

Ross DE, Brantley SL, Tien M (2009) Kinetic characterization of OmcA and MtrC, terminal reductases involved in respiratory electron transfer for dissimilatory iron reduction in *Shewanella oneidensis* MR-1. Appl Environ Microbiol 75:5218–5226

Shi L, Dong H, Reguera G, Beyenal H, Lu A, Liu J, Yu HQ, Fredrickson JK (2016) Extracellular electron transfer mechanisms between microorganisms and minerals. Nat Rev Microbiol 14:651–662

Wu Y, Liu T, Li X, Li F (2014) Exogenous electron shuttle-mediated extracellular electron transfer of *Shewanella putrefaciens* 200: electrochemical parameters and thermodynamics. Environ Sci Technol 48:9306–9314