Kinetics and Mechanism of Mineral Respiration: How Iron Hemes Synchronize Electron Transfer Rates

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Abstract: Anaerobic microorganisms of the Geobacter genus are effective electron sources for the synthesis of nanoparticles, for bioremediation of polluted water, and for the production of electricity in fuel cells. In multistep reactions, electrons are transferred via iron/heme cofactors of c-type cytochromes from the inner cell membrane to extracellular metal ions, which are bound to outer membrane cytochromes. We studied ET processes, where mediated by proteins of bacterial filaments or by inside of the cells to the exterior. ET reactions can be for bioremediation of toxic and radioactive metals, and for the production of nanoparticles, which are effective electron sources for the synthesis of nanoparticles, and for bioremediation of polluted water, and for the production of electricity in fuel cells. In multistep reactions, electrons are transferred via iron/heme cofactors of c-type cytochromes from the inner cell membrane to extracellular metal ions, which are bound to outer membrane cytochromes. We measured electron production and electron flux rates to $5 \times 10^6$ es$^{-1}$ per G. sulfurreducens. Remarkably, these rates are independent of the oxidants, and follow zero order kinetics. It turned out that the microorganisms regulate electron flux rates by increasing their Fe$^{2+}$/Fe$^{3+}$ ratios in the multiheme cytochromes whenever the activity of the extracellular metal oxidants is diminished. By this mechanism the respiration remains constant even when oxidizing conditions are changing. This homeostasis is a vital condition for living systems, and makes G. sulfurreducens a versatile electron source.

Some anaerobic microorganisms are able to use extracellular metal salts as terminal oxidants in their respiratory pathways (mineral respiration). Important examples are bacteria of the Geobacter genus,[1] which turned out to be effective electron sources for the synthesis of nanoparticles,[2] for bioremediation of toxic and radioactive metals,[3] and for the current production in microbial fuel cells.[4] The respiratory pathways are based on electron transfer (ET) from the inside of the cells to the exterior. ET reactions can be mediated by proteins of bacterial filaments or by c-type cytochromes.[1b-7] We studied ET processes, where c-type cytochromes are the electron carriers (Figure 1). The respiratory chain starts at the inner cell membrane (electron influx) by enzymatic reduction of the Fe$^{3+}$/heme cofactors of inner membrane cytochromes (Imc). During electron transport through the periplasm, several ET steps occur within and between the periplasmic multiheme cytochromes (Ppc). Finally, Fe$^{3+}$/hemes of the outer membrane cytochromes (Omc) accept these electrons, and reduce the extracellular metal ions (electron outflux).

We have measured the electron production and flux through the cell by in vivo experiments with Geobacter sulfurreducens using AgNO$_3$, Na$_2$CrO$_4$, and AgCl as oxidants (Scheme 1). It turned out that each microorganism produces $5 \times 10^6$ electrons per second, and that the electron flux rate is nearly independent of oxidation potentials, aggregation states, charges and concentrations of the extracellular metal salts. This surprising behavior is accomplished by the bacteria, which are able to vary the Fe$^{3+}$/Fe$^{2+}$ ratios in the hemes of the cytochromes, allowing to synchronize the electron outflux rates with the constant electron influx rate.
First experiments were performed with AgNO₃, which was reduced by G. sulfurreducens to Ag nanoparticles (AgNPs). Redox reactions between the oxidant and the Fe²⁺/hemes rely on complexation of Ag⁺ ions by the amino acids of Omc proteins. High complexation constants prevent the migration of Ag⁺ into the cell, and thus protect the bacteria against the toxicity of Ag⁺ ions. To verify Ag⁺ complexation, NMR titration experiments were carried out with OmcF, the only Omc of G. sulfurreducens whose structure was determined in NMR studies.[8] In the 11 kDa OmcF a methionine, Met86, and a histidine, His34, are well oriented for the coordination of a metal ion in a linear fashion (Figure 2A). Both amino acids are known to bind Ag⁺ ions in peptides.[9] ¹H NMR titration of OmcF with silver nitrate in D₂O confirmed silver complexation by the imidazole group of His34 as well as by the sulfur atom of Met86 (Figure S1).

This titration furthermore indicated the formation of a 3:1 Ag⁺/OmcF complex (Figure 2B), implying other coordinating residues whose proton signals could not be followed, likely because of the neighboring paramagnetic iron/heme entity. In order to determine the binding constant, we added imidazole-d₄ as competing ligand, whose affinity constant to Ag⁺ ions is known.[10] This created two sets of protein signals (Figures 2C and Figure S2), which were due to a partial exchange of the axial His39-heme ligand by the added imidazole-d₄. This type of exchange had already been observed in other iron/heme systems.[11] and we confirmed this ligand exchange for OmcF by variation of the imidazole-d₄ concentration (Figure S3). From the signals of Met86 and His34, two of the three Ag⁺/OmcF binding constants were determined to be logK₁,₁ = 6.1(±0.3) and logK₂,₁ = 6.2(±0.5) (Figure S4). The third complexation could not be fitted with a reasonable error. The binding constants correspond to typical values reported for other histidine/silver/methionine complexes.[10,12] Binding between Ag⁺ and two (protein-free) histidine molecules has also a binding constant on the order of 10⁶[10] The NMR structures of the outer membrane cytochromes of G. sulfurreducens such as OmcB and OmcC are not known. They are large, multiheme cytochromes with a high number of free histidine residues, which should bind Ag⁺ ions also very efficiently.[13]

Experiments to determine the ET rates were carried out with 0.6–0.9 µM G. sulfurreducens and 50 µM AgNO₃ solutions with low Cl⁻ and Fe²⁺ concentrations (Figure S5).[14] UV/Vis spectra exhibited Soret and Q-bands of the iron hemes, as well as scattering contributions of the microorganisms (Figure 3A). As the AgNP concentration was low, the plasmon resonance of the AgNPs was weak and overlapped with the iron/heme Soret bands (Figure S6). Therefore, the concentration changes of the Fe²⁺/hemes were analyzed by their Q-bands (Figure S7). AgNP formation was determined by the increasing scattering of the microorganisms at 610 nm (Figure 3C.D)[15] and EDS experiments (Figure S8).

Addition of AgNO₃ to G. sulfurreducens oxidized in less than two seconds more than 98% of the Fe²⁺/hemes to Fe³⁺/hemes (Figure S9), and the Fe³⁺/Fe²⁺ ratio remained nearly constant for the next 4–5 minutes (Figure 3B). During this time, the light scattering increased in a nearly linear way (Figure 3C), which indicated a constant AgNP formation that was independent of the Ag⁺ concentration.[15] Thus, its rate
followed zero order kinetics. After consumption of the Ag\(^+\) ions, Fe\(^{3+}\)/hemes were reduced back to Fe\(^{2+}\)/hemes (Figure 3B). The observed zero order kinetics during AgNP formation can be explained by a constant number of reaction sites at which AgNPs grew on the surface of the bacteria (Figure 3D), as well as an excess of Ag\(^+\) sites at which AgNPs grew on the surface of the bacteria formation can be explained by a constant number of reaction steps between and through the \(c\)-type cytochromes. Thus, the ET steps between and through the \(c\)-type cytochromes leading to 4, which continues the AgNP growth.

**Scheme 2.** Formation of AgNPs starts with complexation of Ag\(^+\) ions to Omc 1 and subsequent oxidation of Fe\(^{3+}\)/hemes to Fe\(^{2+}\)/hemes in 2. The AgNP growth occurs by Ag\(^+\) addition (2→3), as well as ET steps between and through the \(c\)-type cytochromes leading to 4, which continues the AgNP growth.

**Table 1:** Electron flux rates with different oxidants and different generations of G. sulfurreducens.

<table>
<thead>
<tr>
<th>Oxidant</th>
<th>Redox potential ([V]) (pH 7)</th>
<th>ET rate ([es^1][h]^{-1})</th>
<th>Temperature ([^\circ C])</th>
</tr>
</thead>
<tbody>
<tr>
<td>AgNO(_3)</td>
<td>0.80</td>
<td>3.0×10(^{-5})</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.5×10(^{-5})</td>
<td>20</td>
</tr>
<tr>
<td>Na(_2)CrO(_4)</td>
<td>0.62</td>
<td>0.8×10(^{-5})</td>
<td>15</td>
</tr>
<tr>
<td>AgCl</td>
<td>0.21</td>
<td>5.0×10(^{-5})</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7.5×10(^{-5})</td>
<td>30</td>
</tr>
</tbody>
</table>

[a] Standard reduction potentials against NHE were used. They were not corrected for the influence of the reaction mixture and the reaction temperature. [b] The variation was ±40%.

A characteristic of the silver ion system is that not the decrease of the oxidants, the Ag\(^+\) ions, but the increase of the reaction products, the AgNPs, was determined. In order to analyze directly the change of the oxidants by UV/Vis spectroscopy, we used Na\(_2\)CrO\(_4\) as a reagent (Figure 4A,C). Another difference in these two oxidants is that CrO\(_4^{2-}\) anions instead of Ag\(^+\) cations were bound by the amino acids of the Omc proteins, and Cr\(^{3+}\) cations instead of Ag\(^0\) nanoparticles were formed as reaction products. Several experiments with 0.7–0.9 pm G. sulfurreducens and 0.05 or 0.1 mm Na\(_2\)CrO\(_4\) solutions at 30\(^\circ\)C were carried out. Figure 4C shows that the concentration of the oxidant decreased in a constant way. Thus, also with Na\(_2\)CrO\(_4\), a concentration change did not influence the redox rate. The reaction was of zero order in chromate, so that its rate decreased to 20 and 15\(^\circ\)C, the electron flux slowed down by a factor of 2.0 and 3.8, respectively.

Interestingly, the initial oxidation of the Fe\(^{3+}\)/hemes of G. sulfurreducens in the resting state by Ag\(^+\) ions (1→2) was much faster than the electron flux during respiration. It took less than 2 seconds to oxidize nearly all Fe\(^{3+}\)/hemes to Fe\(^{2+}\)/hemes (Figure S9). During this initial phase, about 10% of the AgNPs were generated. As Ag\(^+\) ions were in \(7×10^7\) fold excess to G. sulfurreducens, the reduction of 10% of the Ag\(^+\) ions was caused by \(0.7×10^7\) Fe\(^{3+}\)/hemes. This number of iron/hemes per bacterium is in good agreement with direct iron/heme measurements by Esteve-Núñez et al.[38]
potentials induced by Cr$^{3+}$ ions attached to amino acids of Omc.

A much more pronounced increase of the Fe$^{2+}$/heme concentration was observed when solid AgCl nanocrystals served as the oxidant.\[3a\] AgCl nanocrystals were generated by addition of AgNO$_3$ to a Cl$^-$/CO$_3^-$-containing growth medium (Figure S10),\[20\] to which the same volume of a bacteria suspension was injected, leading to a 0.1 mM AgCl and 0.45 mM G. sulfurreducens solution. Within 5 minutes (Figure 5A,C) the microorganisms reduced the insoluble AgCl nanocrystals (Figure 5D) into insoluble AgNPs (Figure 5E).\[21\] The AgNP formation rate remained constant, demonstrating that the product formation again followed a zero order kinetics. However, in sharp contrast to experiments with soluble Ag$^+$ ions (Figure 3B), the reduction of solid AgCl was accompanied by an increase of the Fe$^{2+}$/heme concentration from 15 to 95% (Figure 5B). This implies different reaction mechanisms. With soluble Ag$^+$ ions as the oxidant, complexation by Omc proteins led to a certain number of AgNPs at the microorganisms. These are the reaction sites to which the large excess of Ag$^+$ ions diffused and became reduced.

The situation with insoluble AgCl nanocrystals as oxidants was completely different as nearly all Ag$^+$ ions were stored in the solid AgCl nanocrystals.\[22\] The reduction of each AgCl particle to an AgNP therefore decreased the concentration of the redox sites, thus the electron flux rate should decrease continuously. However, Figure 5C shows that the rate of the AgNP growth remained constant. This was actually accomplished by the concentration increase of the reaction partners, the Fe$^{2+}$/hemes (Figure 5B). Obviously, the increasing Fe$^{2+}$/heme:Fe$^{3+}$/heme ratio in a multiheme Omc raised its reactivity to such an extent that the AgNP formation rates remained constant. We measured an average ET rate at 30°C of 7.5 $\pm$ 10$^5$ es$^{-1}$ per bacterium with a variation of 40% (Table 1). The chemical rates in Table 1 show that respiration of G. sulfurreducens using different oxidants occurs with EET rates of 5 $\pm$ 2 $\times$ 10$^5$ es$^{-1}$ per bacterium at 30°C. This corresponds to a current production of 80 $\pm$ 30 fA, which is similar to electrochemical data. Jian et al. have measured a current of 90 $\pm$ 30 fA in experiments where a single G. sulfurreducens cell was in contact with a gold electrode,\[23\] and El-Naggar et al. determined currents of 60 $\pm$ 40 fA in electrochemical measurements with Shewanella oneidensis.\[24\]

Our experimental results show that in mineral respiration of G. sulfurreducens, the electron flux remains constant during the decrease of the oxidant concentration, demonstrating zero order reaction kinetics. In addition, the electron...
flux rates are nearly independent of the type of the oxidant. This could be a general effect during mineral respiration of anaerobic bacteria; it might occur also with other members of the Geobacter genus or with S. oneidensis microorganisms. The independence from the environmental conditions demonstrates the flexibility of these bacteria, which adapt to changing external redox potentials by changing Fe\(^{3+}/\)heme:Fe\(^{2+}/\)heme ratios in the c-type cytochromes. Decreasing the reactivity or the number of the reaction sites of the oxidizing metal ions is compensated by an increase of the Fe\(^{2+}/\)heme population in the multiheme outer membrane cytochromes. A constant ET is needed to maintain constant ATP synthesis during respiration.\[^{[25]}\] Such ATP homeostasis is a vital condition for living systems.\[^{[26]}\] Obviously, the Fe\(^{3+}/\)heme concentrations in the inner membrane cytochromes remain high enough to stabilize the enzymatic respiration reaction. This might be one of the reasons for the high concentration of iron/hemes in these microorganisms (10\(^7\) per G. sulfurreducens). Thus, ATP homeostasis is the result of an a predictable reaction tool for redox reactions.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: electron transfer - Geobacter sulfurreducens - iron heme - silver nanoparticles - silver proteins


\[^{[13]}\] a) UniProtKB-B7494K(CYCB_GEOSL); b) UniProtKB-Q749L1 (CYTC_GEOSL).

\[^{[14]}\] In a study using different AgNO\(_3\) amounts these concentrations turned out to be appropriate for detailed experiments. The preparation of the G. sulfurreducens solution followed the procedure described in S. I. Vasylevskyi, S. Kracht, P. Corcosa, K. M. Fromm, B. Giese, M. Fuegg, Angew. Chem. Int. Ed. 2017, 56, 5926 – 5930; Angew. Chem. 2017, 129, 6020 – 6024. The growth medium is given in Figure S5. The experiments were carried out under strictly anaerobic conditions. The OD\(_{600}\) values for different G. sulfurreducens generations varied between 0.45 and 0.75. Dilution experiments showed that OD\(_{600}\) changed in a linear way. The kinetic experiments were carried out in 3.0 mL of thermostatted G. sulfurreducens suspensions to which 0.1 mL AgNO\(_3\) solutions were added with a syringe, so that the concentration in the reaction mixture was 0.05 mm. The concentrations of the Cl\(^-\) and Fe\(^{2+}\) ions in the solutions were 2 and about 10 mm, respectively. The Fe\(^{3+}/\)heme concentrations were determined by integration of the Q-band area.

\[^{[15]}\] We detected that the intensity at 610 nm increased linearly in experiments with increasing Ag\(^+\) concentration. We checked the Ag\(^+\) concentrations in the mother liquor by ICP spectroscopy: when the intensity increase had stopped, Ag\(^+\) ions in the mother liquor could no longer be detected, and the analysis during the redox reaction showed a decrease in the Ag\(^+\) concentration. Thus, we concluded that this intensity change might be an acceptable way to measure the formation of AgNPs. We decided
to use this observable, because ICP experiments were not very precise, as the bacteria had to be removed by filtration or centrifugation. Both procedures took time during which the microbiological reaction continued. In addition, during filtration some Ag⁺ ions might be removed by the bacteria in the filter pores. A further complication was that the redox process were fast, requiring only a few minutes (Figure 3), which caused large errors in the ICP experiments. Therefore, we used the intensity at 610 nm to measure the formation and growth of AgNPs. In future work we will further study this phenomenon.

[16] Complexation of Ag⁺ ions by Omc at the beginning of the experiment generated the AgNP growth sites. Analyses of several TEM pictures showed that the number of AgNPs number is always less than 10⁵ per microorganism (see, for example, Figure 3D). The excess of the Ag⁺ ions per microorganism at the beginning of the experiment was 0.7 × 10⁷.

[17] From the rate coefficient of 0.5 × 10⁻¹⁰ L mol⁻¹ s⁻¹ for the addition of Ag⁺ ions to neutral Ag⁺ one can assume that also the reaction with AgNPs might be very fast, see: A. Henglein, *Chem. Rev.* 1989, 89, 1861–1873; A. Henglein, *Ber. Bunsen-Ges.* 1977, 81, 556–561. ET reactions through the multiheme cytochrome MtrF of *Shewanella oneidensis* had been calculated to be about 10⁴ s⁻¹: M. Breuer, K. M. Rosso, J. Blumberger, *Proc. Natl. Acad. Sci. USA* 2014, 111, 611–616. ET between the iron/hemes of different cytochromes should be even slower.


[19] The kinetic experiments were carried out as described for AgNO₃ reactions. Under strictly anaerobic conditions, 0.1 mL Na₂CrO₄ solutions were added to the bacterial suspension in such concentrations that the chromate concentrations were 0.05 or 0.1 mM. Variation of the Cl⁻ concentration had no effect on the EET rate.

[20] Under strictly anaerobic conditions, 0.05 mL of a 0.3 M KCl solution was combined with 1.35 mL of the growth medium. Injection of 0.1 mL of a 3 mM AgNO₃ solution generated AgCl nanocrystals, as powder X-ray measurements proved (Figure 5D and Figure S9). After a few seconds, the mixture became clear and was combined with 1.5 mL of a suspension of *G. sulfurreducens* in the resting state.

[21] Using the dimensions of the AgCl unit structure, the number n of Ag⁺ ions in roundish AgCl nanocrystals with a diameter d is n = 12·d⁴. The number of Ag⁺ in AgNPs is n = 30·d⁴, see: A. Henglein, M. Giersig, *J. Phys. Chem. B* 1999, 103, 9533–9539.

[22] A medium-sized (20 nm) AgCl nanocrystal contains about 10⁵ Ag⁺ ions.


The bacterium *Geobacter sulfurreducens* requires extracellular metal salts for respiration. Each microorganism produces $5 \times 10^5$ electrons per second, which reduce the metal ions bound to the outer membrane cytochromes. With iron heme cofactors of $c$-type cytochromes, the electron flow is independent of the concentration and type of oxidant. This results in ATP homeostasis.