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Electron transfer between hydrogenases and mono- and multiheme cytochromes in *Desulfovibrio* ssp

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Abstract A comparative study of electron transfer between the 16 heme high molecular mass cytochrome (Hmc) from *Desulfovibrio vulgaris* Hildenborough and the [Fe] and [NiFe] hydrogenases from the same organism was carried out, both in the presence and in the absence of catalytic amounts of cytochrome c_3. For comparison, this study was repeated with the [NiFe] hydrogenase from *D. gigas*. Hmc is very slowly reduced by the [Fe] hydrogenase, but faster by either of the two [NiFe] hydrogenases. In the presence of cytochrome c_3, in equimolar amounts to the hydrogenases, the rates of electron transfer are significantly increased and are similar for the three hydrogenases. The results obtained indicate that the reduction of Hmc by the [Fe] or [NiFe] hydrogenases is most likely mediated by cytochrome c_3. A similar study with *D. vulgaris* Hildenborough cytochrome c_{553} shows that, in contrast, this cytochrome is reduced faster by the [Fe] hydrogenase than by the [NiFe] hydrogenases. However, although catalytic amounts of cytochrome c_3 have no effect in the reduction by the [Fe] hydrogenase, it significantly increases the rate of reduction by the [NiFe] hydrogenases.

Key words *Desulfovibrio* · Cytochrome *c* · Hydrogenase · Electron transfer

Abbreviations *Dv*H *Desulfovibrio vulgaris* Hildenborough · *Dv*M *Desulfovibrio vulgaris* Miyazaki · *Dg Desulfovibrio gigas* · *Hmc* high molecular weight cytochrome · *H_2ase* hydrogenase · c_3 cytochrome c_3 · c_{553} cytochrome c_{553}

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Introduction

Sulfate reducers are a group of microorganisms, comprising both bacteria and archaea, which derive energy from the anaerobic respiration of sulfate. They are capable of using lactate, pyruvate, formate and other organic compounds as electron donors, and can even grow chemolithotrophically using hydrogen as the electron donor. Many studies have been carried out on the metabolism of these bacteria [1], as well as on the characterization of their protein components which include a vast array of very interesting and unique redox enzymes and electron carriers [2]. However, several fundamental questions still remain to be elucidated, e.g. what is the actual mechanism for energy conservation in these organisms? Or in other words, what is the mechanism by which the reducing power derived from the oxidation of the carbon substrates is transferred to the reduction of sulfate, leading to oxidative phosphorylation? In particular, and contrary to other types of organisms, the terminal reductases involved in sulfate reduction are not membrane-bound, but cytoplasmic, and so are not directly involved in proton translocation across the membrane.

A hydrogen cycling mechanism was proposed by Odom and Peck [3] as the general mechanism for energy conservation in *Desulfovibrio*, the best studied genus of the sulfate reducers [4] (Fig. 1). In this mechanism, the reducing power produced in the oxidation of the carbon substrates is used by a cytoplasmic hydrogenase to form hydrogen, which then diffuses across the membrane to the periplasm, where it is oxidized by a periplasmic hydrogenase. The protons translocated by this process are used to activate the ATP synthase, whereas the electrons return to the cytoplasm for the reduction of sulfate, via a membrane-bound electron-transfer chain. This mechanism has been much debated and some arguments have been put forward against its operation [1, 5], the main one being the fact that there is no evidence for the existence of a cytoplasmic hydrogenase in all species of *Desulfovibrio*. Nevertheless,

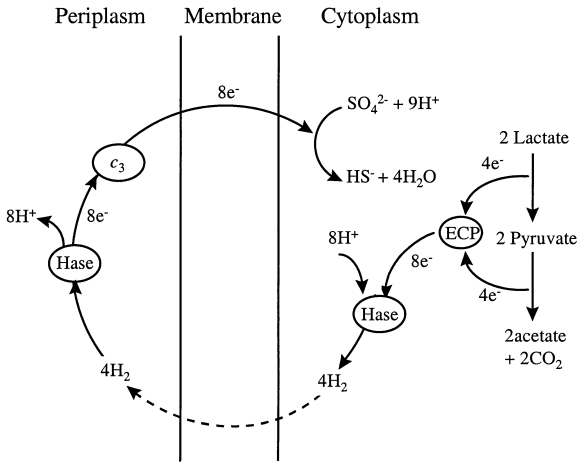

Fig. 1 The hydrogen cycle model [3]. (*H_2ase* hydrogenase, *ECP* electron carrier proteins, *c_3* cytochrome c_3)

several *Desulfovibrio* organisms (*D. vulgaris* Marburg and Hildenborough, *D. gigas* and *D. desulfuricans* Essex 6) can be grown using hydrogen as the sole energy source [6], indicating that the oxidation of hydrogen linked to the reduction of sulfate is an energy-conserving process.

Three different types of hydrogenases (H_2ases) have been characterized from various species of *Desulfovibrio*: the Fe-only ([Fe]), the nickel-iron ([NiFe]) and the nickel-iron-selenium ([NiFeSe]) [7]. These can be distinguished in terms of their metal content, type of redox centres, amino acid sequence, sensitivity to inhibitors and immunological reactivities. In some species of *Desulfovibrio* only one type of hydrogenase has been detected, whereas others have two or three types. A screening of 25 species showed that the gene for the [NiFe] hydrogenase is present in all of the screened species, whereas the presence of genes for the [Fe] and [NiFeSe] enzymes was only demonstrated in some of them [8]. As examples, *D. vulgaris* Hildenborough (*Dv*H) contains the three types of hydrogenase, *D. fructosovorans* contains the [Fe] and [NiFe] hydrogenases and *D. vulgaris* Miyazaki F (*Dv*M F) contains the [NiFe] and [NiFeSe] enzymes, whereas *D. gigas* (*Dg*) contains only a periplasmic [NiFe] hydrogenase [8]. In this last species a bioenergetic mechanism different from the hydrogen cycling hypothesis may be operating, or a completely distinct hydrogenase remains to be found.

Whether or not hydrogen is involved as an intermediate, the mechanism of energy conservation in *Desulfovibrio* must involve charge separation through a membrane-bound electron-transfer chain. A likely candidate for electron transport through the membrane was discovered when cloning the gene of the high molecular mass cytochrome (Hmc) in *Dv*H. It was found that this gene was part of a large operon coding for a transmembrane protein complex containing several redox proteins [9]. Hmc is a 65-kDa cytochrome containing 16 *c*-type hemes, of which 12 hemes are organized in three tetraheme cytochrome c_3-like domains, another three in a triheme cytochrome c_3-like domain [12] and the last one outside of these domains. It has been isolated from *D. vulgaris* strains Hildenborough [10] and Miyazaki F [11], and also from *D. gigas* [12]. Although Hmc is not a hydrophobic protein, it can be isolated in higher amounts from the membranes than from the soluble fraction, suggesting that it is attached to the membrane through the other proteins of the transmembrane redox complex [13]. The other open reading frames in the *Dv*H Hmc operon code for the following putative proteins: HmcB (40 kDa) which contains a typical motif for iron-sulfur clusters located in the periplasmic side of the membrane [14], HmcC (43 kDa), HmcD (6 kDa) and HmcE (25 kDa) which are all putative integral membrane proteins, and HmcF (53 kDa) which is a putative membrane-associated cytoplasmic protein also containing iron-sulfur clusters.

Recent evidence suggests that the Hmc complex is involved in the metabolism of hydrogen, but not in the metabolism of lactate or pyruvate [15], indicating that its role is most probably to transfer electrons formed by a periplasmic hydrogenase across the membrane for the cytoplasmic process of sulfate reduction. Given the similarity between Hmc and three cytochrome c_3 molecules, and the fact that *Dv*H contains three types of hydrogenase, it was proposed that in *Dv*H each of the three Hmc domains could interact specifically with each of the hydrogenases [9]. However, this hypothesis was later discarded [16] due to the fact that Hmc was isolated from *Dg*, which has only the periplasmic [NiFe] hydrogenase.

In view of all these findings it was decided to undertake a study of the reduction of *Dv*H Hmc with the periplasmic [Fe] hydrogenase and the recently purified membrane-bound [NiFe] hydrogenase [17], and also of the effect of cytochrome c_3 in these reductions. For comparison, the *Dg* periplasmic [NiFe] hydrogenase was also studied.

Several kinetic studies have been carried out on the electron transfer between hydrogenases and cytochromes from various sulfate-reducing organisms. In most studies the second order rate constants were determined by electrochemical methods, and in some cases the K_m values were determined by spectrophotometry [18–22].

Materials and methods

Protein purification

*Dv*H cytochrome c_3 [23], *Dv*H Hmc [13], *Dv*H cytochrome c_{553} [24], *Dv*H [Fe] hydrogenase [25], *Dv*H [NiFe] hydrogenase [17] and *Dg* [NiFe] hydrogenase [26] were all purified as previously described. The protein concentrations were determined from their visible spectra using the following absorption coefficients: cytochrome c_3 116 mM^{-1} cm^{-1} (552 nm, reduced); Hmc, 428 mM^{-1} cm^{-1} (552.5 nm, reduced); cytochrome c_{553}, 30 mM^{-1} cm^{-1}

(553 nm, reduced); hydrogenases, 46 mM^{-1} cm^{-1} (400 nm, oxidized).

Enzymatic measurements

All experiments were performed with a Shimadzu UV3100 spectrophotometer, in a stirred cell, with a hydrogen overpressure of 15 kPa flowing through the cell. The buffer used in all cases was 50 mM Tris-HCl, pH 7.6. The reduction of the cytochromes was measured by following the increase in absorption at 552 nm for cytochrome c_3, 552.5 nm for Hmc and 553 nm for cytochrome c_{553}, using the respective absorption coefficients. The rates were measured from the linear portion of the reduction curves. The concentrations used were chosen so that a reasonable rate could be measured, having assured that the rates were proportional to the hydrogenase concentrations. Each of the experiments was repeated at least three times. The experimental procedure was different for each type of hydrogenase, because the results obtained when the [NiFe] hydrogenases were used inside a glove box were erratic and unreproducible, whereas the [Fe] hydrogenase was unstable when kept under hydrogen in the gas-tight line. For this reason the rates observed should only be directly compared for the same hydrogenase. It must also be remembered that the activity of each hydrogenase depends on its activation behaviour, which is influenced by several factors like the concentrations of oxygen and of cytochrome c_3, and has not been elucidated for the case of the [Fe] hydrogenase.

Reductions with the *Dv*H [Fe] hydrogenase

For all experiments with the *Dv*H [Fe] hydrogenase, the reaction mixture containing the buffer, the cytochrome to be reduced and the hydrogenase was prepared inside an anaerobic glove box (Mbraun MB 150 GI) with an argon atmosphere, keeping the oxygen level below 0.4 ppm. The cell was closed with a rubber cap before being removed from the glove box. The experiment was started by inserting needles through the rubber cap and letting hydrogen flow through the cell. The concentrations used in each experiment were: Hmc/H_2ase, 0.5 μM Hmc and 0.56 μM H_2ase; Hmc/H_2ase/c_3, 1 μM Hmc, 28 nM H_2ase and 28 nM cytochrome c_3; c_3/H_2ase, 4 μM cytochrome c_3 and 2.8 nM H_2ase; c_{553}/H_2ase, 4 μM cytochrome c_{553} and 28 nM H_2ase; c_{553}/H_2ase/c_3, 4 μM cytochrome c_{553}, 28 nM H_2ase and 28 nM cytochrome c_3.

Reductions with the [NiFe] hydrogenases

Both [NiFe] hydrogenases had to be activated before performing the experiments. This was achieved by flushing the H_2ase with hydrogen for about 1 h, and then leaving them overnight at 4 °C under a hydrogen atmosphere. For the experiments in which catalytic amounts of cytochrome c_3 were used, the activation of the H_2ases was performed after adding the cytochrome. A slight pink colour due to reduced cytochrome c_3 could be observed in those cases, indicating that the H_2ase was activated and the solution anaerobic. The experiments were carried out using a vacuum manifold with a flow of hydrogen. The cytochrome and buffer solutions were deoxygenated by performing several cycles of vacuum/hydrogen. The H_2ase solutions were maintained under a flow of hydrogen. The cell was evacuated and flushed with hydrogen. The buffer and cytochrome solutions were transferred to the cell under a flow of hydrogen, using gas-tight syringes. Small volumes of a dithionite solution (1–5 μl) were added to remove traces of oxygen until a minimal stable state of cytochrome reduction was observed. At this point the H_2ase was added with a gas-tight syringe and the reduction of the cytochrome was followed. The concentrations used in each experiment were: Hmc/H_2ase, 1 μM Hmc and 130 nM H_2ase; Hmc/H_2ase/c_3, 1 μM Hmc, 130 nM H_2ase and 130 nM cytochrome c_3; c_3/H_2ase, 4 μM cytochrome c_3 and 6.5 nM H_2ase; c_{553}/H_2ase, 4 μM cytochrome c_{553} and 130 nM H_2ase; c_{553}/H_2ase/c_3, 4 μM cytochrome c_{553}, 32.5 nM H_2ase and 32.5 nM cytochrome c_3.

Results and discussion

The rates obtained for the reduction of each cytochrome are presented in Table 1. It must be noted that, as discussed in the previous section, the reduction rates of the three cytochromes can only be directly compared for the same hydrogenase.

The reduction of Hmc with the *Dv*H [Fe] hydrogenase alone was the most problematic to be measured. Using catalytic amounts of hydrogenase, variable lag phases, from 15 min to 1 h, were observed, and the reduction rates measured were unreproducible, although always very low. In some experiments, no reduction was observed even after prolonged incubations under hydrogen. To observe a reproducible reduction of this cytochrome by the [Fe] hydrogenase, it was necessary to use a very high concentration of hydrogenase, in fact similar to that of Hmc (0.5 μM Hmc and 0.56 μM H_2ase). This indicates that electron transfer from the [Fe] hydrogenase to Hmc, although possible, is most probably not a physiologically relevant process. For the sake of comparison, the rate of reduction of cytochrome c_3 by the [Fe] hydrogenase was also measured. This rate was at least four orders of magnitude higher than that of Hmc. Since the concentration of cytochrome c_3 in the cells is high, in physiological conditions Hmc will not be able to compete with cytochrome c_3, as an electron acceptor for the [Fe] hydrogenase.

However, when the reduction of Hmc by [Fe] hydrogenase was performed in the presence of a catalytic amount of cytochrome c_3 (equimolar with the hydrogenase) a completely different picture was observed. The lag phase in this case was only of a few minutes, the reduction was consistent and reproducible, and the rate increased by two orders of magnitude. This result suggests that in vivo the reduction of Hmc by periplasmic [Fe] hydrogenase is mediated by cytochrome c_3.

Table 1 Rates obtained for the reduction of each cytochrome from *Dv*H with each of the hydrogenases [(nmol Cyt)·min^{-1}·(nmol H_2ase)$^{-1}$]

	*Dv*H [Fe] H_2ase	*Dv*H [NiFe] H_2ase	*Dg* [NiFe] H_2ase
*Dv*H Hmc	0.03	0.38	0.40
*Dv*H Hmc + *Dv*H c_3	4.3	3.5	6.5
*Dv*H c_3	710	1600	1780
*Dv*H c_{553}	24	2.6	2.4
*Dv*H c_{553} + *Dv*H c_3	27	510	380

This is in agreement with the idea that the three separate domains of Hmc are not designed to interact specifically with each of the *Dv*H hydrogenases [16].

Reduction of Hmc with *Dv*H [NiFe] hydrogenase was not so problematic. Although this hydrogenase is much less active than the [Fe] hydrogenase [7], the experiment could easily be performed with a catalytic concentration of hydrogenase (1 μM Hmc and 130 nM H_2ase), giving appreciable rates for the direct reduction of Hmc. Experiments with the *Dg* [NiFe] hydrogenase gave very similar results. Even so, the rate of cytochrome c_3 reduction with either [NiFe] hydrogenase was over three orders of magnitude higher than that of Hmc alone.

However, when catalytic amounts of cytochrome c_3 are used, the reduction rates of Hmc by the [NiFe] hydrogenases increase, and are similar to the rate observed with the [Fe] hydrogenase. Thus, the in vivo reduction of Hmc by [NiFe] hydrogenase is also likely to be mediated by cytochrome c_3.

Similar experiments were carried out with the monoheme cytochrome c_{553} which has a much higher reduction potential than cytochrome c_3. For cytochrome c_{553} the situation is the reverse of Hmc: cytochrome c_{553} is more efficiently reduced by the [Fe] hydrogenase than by the [NiFe] hydrogenases. Albeit slow, full reduction of cytochrome c_{553} was observed with either [NiFe] hydrogenase, in contrast to our previous observations which indicated that this cytochrome was not reduced by the *Dg* [NiFe] hydrogenase [27] and the observation that in *Dv*M F the reduction of cytochrome c_{553} by the [NiFe] hydrogenases was rather non-reproducible [28]. It should be noted that cytochrome c_{553} is present in large amounts in *Dv*M F which only contains [NiFe] and [NiFeSe] hydrogenases. A catalytic amount of cytochrome c_3 had practically no effect on the rate of reduction of cytochrome c_{553} by the [Fe] hydrogenase, whereas it increased this rate by two orders of magnitude in the case of [NiFe] hydrogenases. This is probably due to the fact that cytochrome c_{553} can on its own act as an electron acceptor for the [Fe] hydrogenase [21].

The physiological role of cytochrome c_{553} is still uncertain. It has been reported to act as an electron acceptor for formate and lactate dehydrogenases [29–31]. It has also been proposed that it could act as the electron acceptor in the oxidation of protoporphyrinogen to protoporphyrin [32]. In this respect, it is interesting that a gene coding for a protein very similar to *Dv*H cytochrome c_{553} is present in the genome of *Helicobacter pylori* at a position adjacent to a gene coding for a coproporphyrinogen III oxidase, and very close to another gene coding for a lactate dehydrogenase [33]. On the other hand, in *Dv*M F the cytochrome c_{553} gene is part of an operon which also encodes a putative protein with strong similarities with the subunit I of cytochrome *c* oxidase from several organisms [34], suggesting that the monoheme cytochrome c_{553} could be its electron donor.

In conclusion, the results obtained indicate that the reduction of Hmc by the [Fe] or [NiFe] hydrogenases is most likely mediated by cytochrome c_3. It has recently been proposed that cytochrome c_3 acts as an energy transducing device (proton thruster) by accepting both protons and electrons produced by the [Fe] hydrogenase, and through its redox-Bohr properties, converting the energy of energized electrons (low redox potential) into energized protons (low pK_a) which can then be used by ATP synthase to drive ATP synthesis [4]. We propose that when hydrogen is used as electron donor, it is oxidized by either the periplasmic [Fe] hydrogenase or the membrane-bound [NiFe] hydrogenase, which then donates the electrons and protons to cytochrome c_3. The de-energized electrons are then passed from cytochrome c_3 to Hmc, which transfers them through the transmembrane redox complex to the cytoplasmic reduction of sulfate, as proposed in [16]. The energized protons are used for ATP synthesis (Fig. 2).

It should be noted that, in vivo, Hmc is bound to the membrane through its association with the transmembrane redox complex, and it cannot be ruled out that this association may affect its electron transfer properties such that it may act as a direct electron acceptor for the hydrogenases. However, since cytochrome c_3 is such an efficient electron acceptor for the hydrogenases and is present in vivo in much higher amounts than Hmc, this possibility seems less likely.

Finally, since Hmc is a very complex protein it is possible that it has other roles besides the above described involvement in the hydrogen metabolism. In particular, it contains two high-spin hemes [13] that may bind a substrate, suggesting an enzymatic role. In the present study, only the reduction of the low-spin hemes of Hmc was followed, but it has previously been reported that the high-spin hemes are also reduced by hydrogenase [12, 13]. It is interesting to note that Hmc

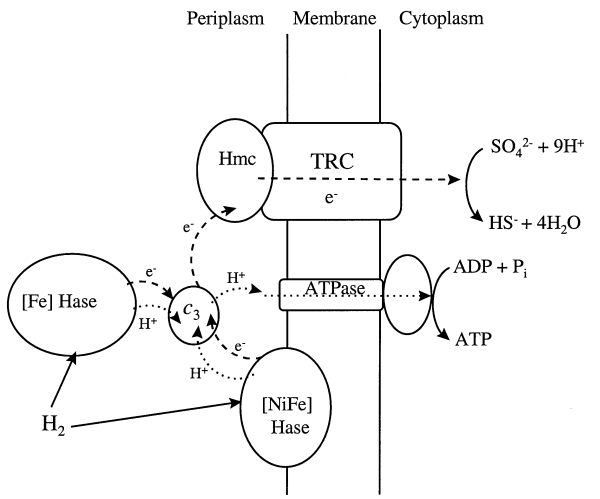

Fig. 2 Proposed electron (*dashed*) and proton (*dotted*) transport pathway during growth of *Dv*H in hydrogen. (*H_2ase* hydrogenase, *c_3* cytochrome c_3, *Hmc* high molecular mass cytochrome, *TRC* transmembrane redox complex [16]; *ATPase* ATP synthase)

has been found in *Dv*H, *Dg* and *Dv*M, species which are very distinct in terms of the type of hydrogenases that they contain. The comparison of the Hmc operon in these species should help to further investigate the involvement of Hmc and its transmembrane complex in the hydrogen metabolism, or in other enzymatic functions.

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