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Diphenylene iodonium as an inhibitor for the hydrogenase complex of *Rhodobacter capsulatus*. Evidence for two distinct electron donor sites

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Abstract

The photosynthetic bacterium *Rhodobacter capsulatus* synthesises a membrane-bound [NiFe] hydrogenase encoded by the H_2 uptake hydrogenase (hup)SLC structural operon. The hupS and hupL genes encode the small and large subunits of hydrogenase, respectively; hupC encodes a membrane electron carrier protein which may be considered as the third subunit of the uptake hydrogenase. In *Wolinella succinogenes*, the hydC gene, homologous to hupC, has been shown to encode a low potential cytochrome *b* which mediates electron transfer from H_2 to the quinone pool of the bacterial membrane. In whole cells of *R. capsulatus* or intact membrane preparation of the wild type strain B10, methylene blue but not benzyl viologen can be used as acceptor of the electrons donated by H_2 to hydrogenase; on the other hand, membranes of B10 treated with Triton X-100 or whole cells of a $HupC^-$ mutant exhibit both benzyl viologen and methylene blue reductase activities. We report the effect of diphenylene iodonium (Ph_2I), a known inhibitor of mitochondrial complex I and of various monooxygenases on *R. capsulatus* hydrogenase activity. With H_2 as electron donor, Ph_2I inhibited partially the methylene blue reductase activity in an uncompetitive manner, and totally benzyl viologen reductase activity in a competitive manner. Furthermore, with benzyl viologen as electron acceptor, Ph_2I increased dramatically the observed lagtime for dye reduction. These results suggest that two different sites exist on the electron donor side of the membrane-bound [NiFe] hydrogenase of *R. capsulatus*, both located on the small subunit. A low redox potential site which reduces benzyl viologen, binds Ph_2I and could be located on the distal [Fe_4S_4] cluster. A higher redox potential site which can reduce methylene blue in vitro could be connected to the high potential [Fe_3S_4] cluster and freely accessible from the periplasm.

Keywords: Hydrogenase; Diphenylene iodonium; Methylene blue; Benzyl viologen; *Rhodobacter capsulatus*

1. Introduction

A number of diaryl iodonium salts, including diphenylene iodonium (Ph_2I), iodonium thiophen and iodonium biphenyl have been shown to strongly inhibit mitochondrial respiration [1,2] and O_2^- production by the oxidase complex of activated neutrophils [3]. In the case of activated neutrophils, it was first reported that the target site of Ph_2I is a flavoprotein of 45 kDa [4]. Later, it was found that at nanomolar concentrations Ph_2I binds predominantly to the haem moiety of a flavocytochrome *b*, which is one of the redox components of the neutrophil NADPH-oxidase complex [5,6]. At higher concentrations, in

Abbreviations: Ph_2I, diphenylene iodonium

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the micromolar range, Ph_2I appears to bind to another site of the flavocytochrome *b*, probably at the level of the flavin component [4,6]. The propensity of Ph_2I to bind the haem moiety of flavocytochrome *b* at nanomolar concentrations is in agreement with the demonstration by Battioni et al. [7] that Ph_2I binds to the haeminic ferrous iron of cytochrome P450. The action mode of Ph_2I on redox enzyme is not fully elucidated, but it seems to happen in two steps. The first one would be the reduction of Ph_2I at a low potential site. This step would be fast and reversible. The reduced Ph_2I is unstable and will produce the iodophenyl radical [2]. This reactive species will later bind covalently to the protein. This step would be generally slower and irreversible.

The hydrogenase of the photosynthetic bacterium *R. capsulatus* belongs to the class of [NiFe] hydrogenases which acts physiologically as a H_2 uptake hydrogenase. Uptake hydrogenase are widespread among eubacteria, ranging from the rumen anaerobic bacterium *Wolinella succinogenes* to the aerobic nitrogen fixing *Azotobacter vinelandii* through the enteric bacterium *Escherichia coli* and the photosynthetic bacteria [8,9] The genes encoding [NiFe] hydrogenases are grouped in clusters. These gene clusters encompassing more than 20 genes, contain not only the hydrogenase structural genes but also a large number of accessory genes necessary for transcriptional regulation, cofactor insertion, protein processing and membrane translocation. In *R. capsulatus*, the structural operon starts with H_2 uptake hydrogenase (hup)S, the gene encoding the hydrogenase small subunit, which is preceded by an unusually long leader sequence of nearly 50 amino acids. The second gene of the operon, hupL, encodes the large subunit [10]. The primary sequence of HupS and HupL presents a high homology with their *Desulfovibrio gigas* counterparts [9] and a total conservation of all the amino acids involved in prosthetic group coordination, i.e. the bimetallic Ni–Fe hydrogen activating site on the large subunit and the three [FeS] clusters of the small subunit [11]. Although the *R. capsulatus* hydrogenase has never been purified in large enough amount to carry out spectroscopic characterisation, we assume that the [FeS] clusters are present and use the same terminology as Volbeda [11]. The $[Fe_4S_4]$ cluster close to the Ni centre is termed 'proximal' and the other one 'distal'.

The structural operon comprises a third gene, hupC, originally called ORFX [12], then hupM [13] lying downstream from hupL. Inactivation of hupC renders the hydrogenase physiologically incompetent so that the HupC protein may be considered as the third subunit of the hydrogenase complex [13]. This organisation is typical for the membrane-bound [NiFe] hydrogenases and the hydrogenase gene products of *R. capsulatus*, *E. coli*, *Ralstorria eutropha* (formerly *Alcaligenes eutrophus*), *A. vinelandii* and *Rhizobium leguminosarum*, share a high degree of identity [14]. In the case of *W. succinogenes*, Dross et al. [15] have presented strong evidence that the product of the third gene hydC is a membrane-bound *b*-type cytochrome which is physiologically reduced by the hydrogenase dimer and can in turn reduces added menaquinone. It is thought that, under physiological conditions, it interacts with the menaquinone pool present in the membrane of *W. succinogenes*. Similarly, the hoxZ gene product has been shown to be a cytochrome *b* [16]. Since the products of *W. succinogenes* hydC, and *R. eutropha* hoxZ gene share a high degree of identity with the deduced product of *R. capsulatus* hupC gene. We assume that this product is a diheam cytochrome *b*.

Since the hydrogenase complex presents a fair amount of low potential redox centres, especially a low potential cytochrome *b*, we decided to investigate the effect of Ph_2I on different preparation of *R. capsulatus* hydrogenase. The results are presented below.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Wild type strain B10 of *R. capsulatus* was obtained from H. Gest (Bloomington, USA). Its $HupC^-$ derivative, the BCX mutant, was constructed by interposon mutagenesis [13]. The presence of a kanamycin resistance gene in hupC renders the hydrogenase of the mutant physiologically incompetent although an active hydrogenase, measurable with artificial dyes, is still produced.

Both strains were grown photoheterotrophically at 30°C under anaerobiosis, using 30 mM DL-malate as carbon and electron source and 7 mM L-glutamate as nitrogen source [17]. Under these conditions, nitro-

genase is expressed, hydrogen gas is produced in large quantities and the synthesis of hydrogenase is induced [14].

2.2. Chemicals

Ph_2I was synthesised by the method of Gatley and Sheratt [1] with slight modification as described by Doussière and Vignais [6]. When solubilised in dimethyl sulfoxide, it exhibits an ultraviolet spectrum with a peak at 264 nm ($\varepsilon_{264} = 15.1$ mM^{-1} cm^{-1}) [2]. All other reagents were of analytical grade.

2.3. Hydrogenase activity assay

Hydrogenase activity was determined spectrophotometrically at 25°C with either methylene blue ($\varepsilon_{630} = 38.7$ mM^{-1} cm^{-1}) [18] or benzyl viologen ($\varepsilon_{555} = 9.5$ mM^{-1} cm^{-1}) [19,20] as electron acceptor and hydrogen as electron donor with Tris–HCl (20 mM, pH = 8.5) as buffer system. These well-established procedures [18,19] are specific of hydrogenase activity. The dye reduction rate was measured under a N_2 atmosphere as a control. With whole cells under exponential growth, a hydrogen independent methylene blue reduction was sometimes observed and corresponds to less than 5% of the hydrogen dependent rate. This baseline activity was subtracted to evaluate the true hydrogenase activity. With cell free extracts, the hydrogen independent activity was essentially zero. The reaction was carried out in an anaerobic cuvette equipped with a side-arm for enzyme incubation and a rubber stopper for direct injection. Typically, the cuvette was filled with 1 ml of buffer containing the electron acceptor and flushed with H_2 or N_2 gas (1 atm. 30 ml min^{-1}) for 10–15 min. When membrane fractions were analysed, 2 to 10 μl was placed in the side-arm. Due to the small volume of the enzyme sample, we observed that the deoxygenation and reductive activation of hydrogenase was complete after 10 min. Alternatively, when the hydrogenase was already in an active state, that is with whole cells under growing conditions or with membranes freshly treated with Triton X-100 and kept under hydrogen, fractions of 2–10 μl were directly injected through the rubber stopper into the hydrogen saturated reaction mixture and the reaction was followed spectrophotometrically for 1–10 min after the lag time.

2.4. Preparation of membrane fraction

Cells of *R. capsulatus* grown photoheterotrophically for 18 h in 1 l Roux bottles ($A_{600} = 2$) were sedimented by centrifugation (10 000 × *g*, 15 min), then resuspended in 20 ml of 20 mM Tris–HCl buffer, pH 8. Cells were disrupted by passage through a French press at 137 MPa, under aerobic conditions. The crude homogenate was centrifuged at 100 000 × *g* for 90 min. The compact pellet of the membrane fraction was resuspended in 20 mM Tris buffer, pH 8, at a final protein concentration of 13.4 mg ml^{-1} and stored at −18°C. Under this storage conditions, the hydrogenase activity was stable for months. A second fraction, termed 'Triton X-100 extract' was obtained by mixing the membrane fraction (2.6 mg protein per ml) with a final 2% (v/v) solution of Triton X-100 for 30 min at 20°C. In the Triton extract, hydrogenase activity was unstable under aerobic conditions. The activity decayed exponentially with a half-life of 24 h. However, under a H_2 atmosphere, hydrogenase activity was stable for at least a week at 20°C, but not at 4°C [17].

2.5. Protein determination

Protein concentration was determined according to the bicinchoninic acid method (Pierce) using bovine serum albumin as a standard.

3. Results

3.1. Partial versus total inhibition

The Ph_2I-dependent inhibition of hydrogenase activity, whatever the fraction used, was almost instantaneous and completed within 30 s (data not shown).

The extent of inhibition was dependent on the bacterial fraction and on the electron acceptor used (Figs. 1 and 2). With methylene blue as oxidant, only a partial inhibition of at most 30% was observed with B10 whole cells (Fig. 2) or with membrane fractions from B10 (data not shown) and 50%

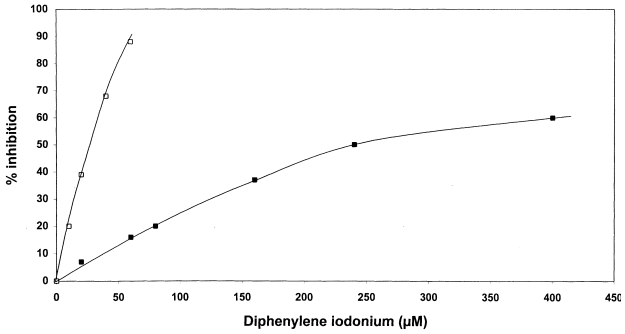

Fig. 1. Inhibition of benzyl viologen reductase activity by Ph_2I. Measurement of hydrogenase activity as described in Section 2. Benzyl viologen reductase activity was measured with 0.5 mM benzyl viologen, (□) in whole cells of BCX1 (200 μl), and (■) in Triton X-100 extracts (2% v/v) of membranes of B10 (26 μg proteins).

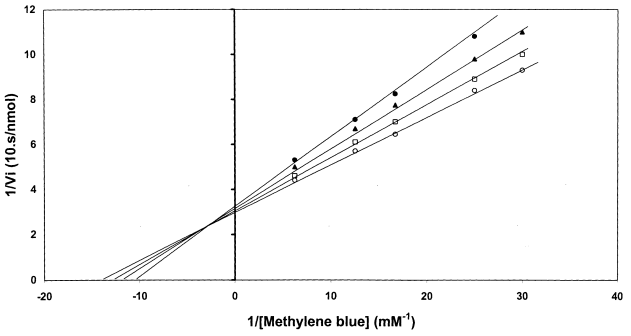

Fig. 3. Double reciprocal plot of methylene blue reductase activity in intact B10 membrane with increasing concentrations of Ph_2I. The concentration of methylene blue varied from 5 to 25 μM. (○) no Ph_2I; (□) 40 μM Ph_2I; (▲) 80 μM Ph_2I; (●) 120 μM Ph_2I.

with BCX mutant whole cells (Fig. 2). On the other hand, total inhibition was obtained when benzyl viologen was used as an electron acceptor. In the BCX mutant cells, the benzyl viologen reductase activity vanished completely at relatively low concentration of Ph_2I (less than 80 μM) (Fig. 1).

3.2. Inhibition pattern with methylene blue as an electron acceptor

3.2.1. Whole cells and intact membranes of B10

With whole cells and with intact membranes of strain B10, only methylene blue and not benzyl viologen can serve as an electron acceptor from H_2 [17]. The series of experiments shown in Fig. 3 was carried out to determine the rate of electron transfer from H_2 to methylene blue, at increasing concentrations of Ph_2I. The same results were obtained with whole cells and with intact membranes of B10. The experimental data are expressed on a double reciprocal plot and present the appearance of a non-competitive inhibition. At this stage, it is not possible to give a mechanistic model based on Segel mixed inhibition nomenclature [21]. These results can be explained by interpreting that Ph_2I acts as a reversible ligand on some site of the hydrogenase complex.

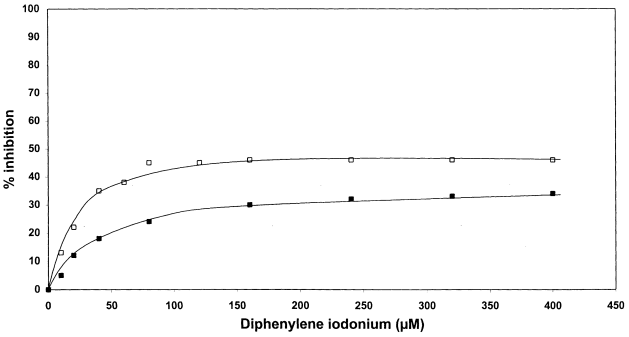

Fig. 2. Inhibition of methylene blue reductase activity by Ph_2I. Measurement of hydrogenase activity as described in Section 2. Methylene blue reductase activity was measured with 40 μM of methylene blue, in whole cells of BCX1 (200 μl) (□), and in whole cells of B10 (200 μl) (■). A similar inhibition pattern was observed with intact membranes of B10 (data not shown).

3.2.2. Triton X-100 extract of B10 membranes and whole cells of the HupC⁻ mutant, BCX1

Since the hydrogenase activity in the HupC⁻ mutant, BCX1, is very labile [13], the experiments with this mutant were carried out with whole cells kept under reducing conditions. Triton X-100 extracts of B10 membranes lost their hydrogenase activity as well but the rate was much slower than with BCX. To maintain hydrogenase activity stable for several hours in order to carry out a complete set of inhibition experiments, the Triton X-100-treated membranes were kept under 1 atm. of H_2 in rubber capped vials at 25°C. Under these conditions, the hydrogenase stayed fully active, and Triton extracts (2–5 μl) could be injected directly inside the H_2-saturated spectrophotometric cuvette. The data relative to BCX whole cells are shown on Fig. 4, using a double reciprocal plot. An uncompetitive type of inhibition is observed with a constant value of the V_m/K_m ratio whatever the Ph_2I concentration used. The apparent K_m value for methylene blue decreased

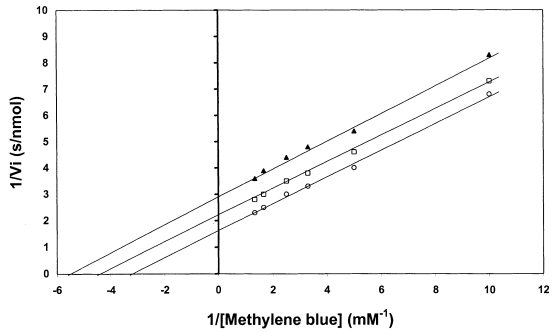

Fig. 4. Double reciprocal plot of methylene blue reductase activity in whole cells of BCX1 with increasing concentrations of Ph_2I. The concentration of methylene blue varied from 10 to 100 μM. (○) no Ph_2I; (□) 160 μM Ph_2I; (▲) 200 μM Ph_2I.

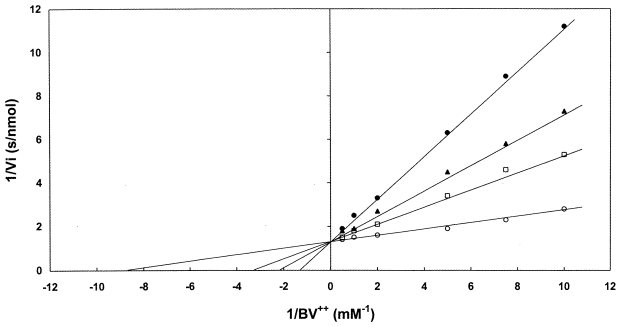

Fig. 5. Double reciprocal plot of benzyl viologen reductase activity of Triton X-100 extract of B10 membrane with increasing concentrations of Ph_2I. The concentration of benzyl viologen varied from 0.1 to 1 mM. (○) no Ph_2I; (□) 80 μM Ph_2I; (▲) 160 μM Ph_2I; (●) 240 μM Ph_2I.

from 28 to 17 μM as Ph_2I increased from 0 to 200 μM.

The same pattern of inhibition was observed with Triton extracts B10 membrane (data not shown). This result is quite different from the non-competitive inhibition obtained with intact membranes of B10 (Fig. 3).

The hydrogenase complex is sensitive to Triton X-100, with a significant alteration of the the methylene blue and Ph_2I binding sites.

3.3. Inhibition pattern with benzyl viologen as electron acceptor

Triton X-100 extract of membranes from B10 and whole cells of the HupC$^-$ BCX1 mutant were used to carried out these experiments. The low potential electron acceptor benzyl viologen ($E^0 = -350$ mV) reacts readily with a large number of cytoplasmic (*Clostridium pasteurianum*) and periplasmic (*D. gigas*) hydrogenases [20]. It does not react, however, with the intact membranes of *R. capsulatus* strain B10 [17]. Benzyl viologen is an effective substrate of *R. capsulatus* hydrogenase only after cell treatment with Triton X-100, or in the case of HupC$^-$ mutant. The Ph_2I inhibition pattern of benzyl viologen reduction by a Triton X-100 extract of B10 cell membranes is shown on Fig. 5, using a double reciprocal plot. The same pattern of inhibition is found in these BCX cells (data not shown). The inhibition is of the competitive type and the calculated K_i values were 12 μM with BCX whole cells and 42 μM with Triton extracts of B10 membranes (Table 1).

3.4. Increase of the lagtime for benzyl viologen reduction in the presence of Ph_2I

A lagtime before dye reduction by hydrogenase is commonly observed, when the enzyme has been isolated under aerobic conditions. From studies on the lagtime observed with the hydrogenase from *D. gigas*

Table 1
Different types of inhibition observed in the *R. capsulatus* hydrogenase activity by Ph_2I

Sample	Triton X-100	Substrate	Activity (nkat/mg protein)	K_m (μM)	K_i (μM)	Inhibition type [21]
Whole cell B10	−	MB	16	18	−	Partial inhibition
Whole cell BCX1	−	MB	33	55	−	Partial inhibition
Whole cell BCX1	−	BV	15	630	12	Competitive Inhibition
Membrane B10	−	MB	40	60	−	Partial inhibition
Membrane B10	+	MB	87	28	−	Partial inhibition
Membrane B10	+	BV	33	90	42	Competitive inhibition

MB: Methylene blue; BV: Benzyl viologen

[20,23,24] it appears that the lagtime depends on the presence of residual molecular oxygen at the enzyme active site and on the redox potential. Some hydrogenases, like the soluble periplasmic enzymes from *Desulfovibrio* species, stay fully inactive in the presence of trace amounts of oxygen [20]. On the other hand, membrane-bound hydrogenases which are part of the respiratory chain in numerous aerobic species can tolerate substantial amounts of molecular oxygen under working conditions. This is the case of the [NiFe]hydrogenase from *A. vinelandii* and the NAD dependent cytoplasmic hydrogenase from *R. eutropha* which withstand 20% oxygen in the gas phase [25].

From a practical point of view, the lagtime may be abolished with improved anaerobic conditions and with active enzyme preparations which require a long preincubation of the enzyme under reducing conditions, for example under dihydrogen gas. The lagtime phenomenon which results from a complex set of biochemical mechanisms seems to be shared by [NiFe] hydrogenases to various degrees. In the case of *R. capsulatus*, the hydrogenase reactivation process is fast in whole cells and in intact membranes, practically completed after 5 min of incubation under dihydrogen gas and, therefore, the lagtime can be easily eliminated. This is probably the functional aerobic respiratory chain present in these biological samples which acts as an efficient oxygen scavenger. No effect of Ph_2I was observed on the lagtime of these preparations.

This was not the case with the mutant BCX or with Triton X-100 extracts of B10 membranes. With methylene blue as electron acceptor, only a slight increase in the lagtime was detected. In sharp contrast, in the presence of benzyl viologen, a dramatic dependency of the lagtime on the concentration of Ph_2I was observed. The effect was more striking with BCX whole cells than with Triton X-100 extracts of B10 membranes (Fig. 6). With BCX whole cells, at Ph_2I concentrations above 80 μM, the lagtime became so long that the measurement of hydrogenase activity was difficult. It should be stressed that under the same experimental procedures, no lagtime was observed in the absence of Ph_2I.

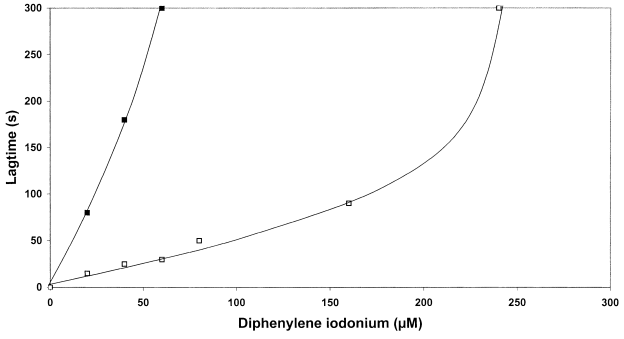

Fig. 6. Increase of the lag time observed with benzyl viologen reduction in presence of Ph_2I. Benzyl viologen 0.5 mM. (■) whole cells of mutant BCX (100 μl); (□) 2% (v/v) Triton X-100 extract of B10 membranes (26 μg protein per assay).

4. Discussion

4.1. Ph_2I acts as a ligand to hydrogenase

Yea et al. [4] using Ph_2I in micromolar concentrations found that Ph_2I binds covalently to a 45 kDa flavoprotein belonging to the superoxide radical-producing complex of activated neutrophils. On the other hand, Doussiere and Vignais, studying also the neutrophil NADPH oxidase report that inhibition of O_2^- production by activated neutrophil membranes is inhibited by nanomolar concentrations of Ph_2I in a time-dependent manner, needs a reduced enzyme and leads to a spectral modification of the cytochrome *b*-558 present in the complex [6]. These results suggest that Ph_2I acts as the sixth ligand of the ferrous atom of cytochrome *b*-558. Interestingly, mono-oxygenases show the same pattern of inhibition by Ph_2I as cytochrome P450 [7]. It appears therefore that one likely site of action of Ph_2I is the reduced ferrous iron of low potential haem proteins.

In the case of *R. capsulatus* hydrogenase, the action of Ph_2I is fast (<10 s) (data not shown) compared to the scale of experimental measurements, and the results reported in Figs. 3–5 best fit with a reaction mechanism in which Ph_2I acts first as a reversible ligand. Ph_2I acts at relatively high concentration. (100 μM range)

4.2. Ph_2I does not interact with the cytochrome but with an iron sulfur cluster

The pattern of hydrogenase inhibition by Ph_2I was

similar with cells of the wild type strain B10 and of the HupC$^-$ mutant BCX when methylene blue was electron acceptor. Since the BCX mutant, which lacks the hupC gene product homologous to the HydC protein of *W. succinogenes*, is still inhibited by Ph_2I, it is concluded that the binding site of Ph_2I is not the HupC protein as anticipated at the beginning of this investigation. On the other hand, the inhibition pattern varies extensively with the type of hydrogenase preparation. It seems unlikely that the bimetallic hydrogen activating centre, deeply buried inside the large subunit [11], would have an accessibility which varied with detergent concentration. Our current hypothesis identifies the low potential site, susceptible to reduce Ph_2I, with the distal [Fe_4S_4] cluster.

4.3. Benzyl viologen and Ph_2I interact at the same site

Ph_2I inhibited completely the dihydrogen-benzyl viologen oxidoreductase activity with two enzyme preparations namely, Triton X-100 extracts of wild type B10 membranes and whole cells of the BCX1 mutant (Fig. 1).

The double reciprocal plot of rates of hydrogenase activity with increasing Ph_2I concentrations indicates that the inhibition is of the competitive type (Fig. 5). Formally, this result only means that the complex hydrogenase–Ph_2I and hydrogenase–benzyl viologen are mutually exclusive. From a practical point of view, it is likely that benzyl viologen and Ph_2I compete for the same site on the hydrogenase complex called the 'viologen site'. It is interesting to note that on intact membranes of the wild type strain B10 the viologen site is not accessible to the viologen dye, however, a short treatment with Triton X-100 or the absence of the hupC gene product (mutant BCX) exposes this site to the external environment. The more likely candidate for the viologen site is the distal [Fe_4S_4] cluster present at the periphery of the small subunit [11].

4.4. Methylene blue and benzyl viologen do not bind to the same site

Benzyl viologen acting as a low potential redox acceptor is an almost universal electron acceptor for hydrogenases [19]. It is readily reduced by purified [NiFe] hydrogenases. The membrane-bound hydrogenase of *R. capsulatus* needs to be solubilised by detergents to exhibit significant benzyl viologen reductase activity [17]. On the other hand, methylene blue, a redox component with a much higher midpoint potential ($E^0 = +11$ mV), is readily reduced or oxidised by numerous components of the respiratory chains. It is not an electron acceptor for the large majority of hydrogenases, especially the periplasmic hydrogenase from *Desulfovibrio* species [20,22] and methanogens but a fairly good acceptor to a small group of membrane-bound uptake hydrogenases. Hydrogenases from *R. capsulatus* and *R. leguminosarum* are representative of this group.

With whole cells or intact membranes of *R. capsulatus* wild type B10 strain, one possible site of dihydrogen-dependent methylene blue reduction is probably at the Complex 3 level of the respiratory chain (ubiquinone–cytochrome *c* oxidoreductase). However, with the *R. capsulatus* system, several lines of evidence indicate the existence of a methylene blue site on the HupSL complex:

1. Treatment of intact membrane by detergents such as Triton X-100 increases the specific activity of methylene blue reductase two to three fold.
2. Delipidation of this solubilised hydrogenase preparation by repeated hexane extractions has no effect on the methylene blue reductase activity (data not shown);
3. The HupC$^-$ BCX mutant which is unable to feed the respiratory chain with H_2 electrons [13], exhibits a normal methylene blue reductase activity.

The inhibition studies carried out on whole cells of B10 and on whole cells of the BCX mutant indicate that Ph_2I is able to only partially inhibit the methylene blue reductase activity (30% in the case of B10 cells and 50% in the case of BCX cells) (Fig. 2).

Furthermore, the inhibition appears non-competitive or competitive in function of the activity measurement procedure. On the other hand, 100% inhibition of benzyl viologen reduction was obtained with Ph_2I with an inhibition of the competitive type. It can be concluded that the hydrogenase possesses a methylene blue site which is different from the viologen site.

4.5. Effect of Ph_2I on the lagtime

As mentioned in Section 3, the lagtime phenomenon is a complex one, depending in part on the residual O_2 concentration, and in part on a reductive activation process of hydrogenase.

The level of molecular oxygen which is tolerable for the determination of hydrogenase activity depends on the enzyme source and on the nature of the electron acceptor. For example, viologen dyes generate a fully anaerobic environment due to the immediate reoxidation by O_2 of reduced viologen. On the other hand, methylene blue or tetrazolium salts allow the presence of O_2 gas in the reaction medium, due to the slower reoxidation of their reduced form by O_2. The source of hydrogenase plays a determinant role in the enzyme behaviour towards molecular oxygen [22]. The reactivation of some hydrogenases is increased by the presence of quinones or the physiologic electrons acceptors. This is the case in the *D. gigas* periplasmic [NiFe] hydrogenase, where the concentration of cytochrome *c*3, that is the natural acceptor for the electrons coming from the hydrogen, increases the rate of enzyme reactivation [23,24,27]. In our case, if the interaction between hydrogenase and benzyl viologen increases the activation rate, the inhibitor, which prevents this interaction, increases the lagtime.

Finally we suggest a model to explain the three kinds of inhibition found (Fig. 7) based on the general topological model proposed by Berks [26].

In fact, some unexpected and interesting observations disclosed a complex situation, with the presence of several interaction sites between the enzyme and its ligands. The three kinds of inhibition, in the model that we propose, are:

1. In the B10 whole cells and intact membranes, (Fig. 7A) the electrons coming from the hydrogen are transferred to the methylene blue ($E^0 = +11$ mV) at a high potential site, possibly at or near the $[Fe_3S_4]$ cluster of HupS.
2. In the BCX cells (Fig. 7C)(where the product of hupC is absent) the viologen site, where Ph_2I can also be fixed, is accessible but it is different from the methylene blue site. So, the HupSL dimer presents two different electron donor sites: one for benzyl viologen and the other one for methylene blue.
3. In the Triton X-100 extract of B10 membranes (Fig. 7B), the detergent can displace the HupC protein, uncovers the viologen site ($E^0 = -360$ mV) and disturbs the ternary structure enough to increase the methylene blue reductase activity.

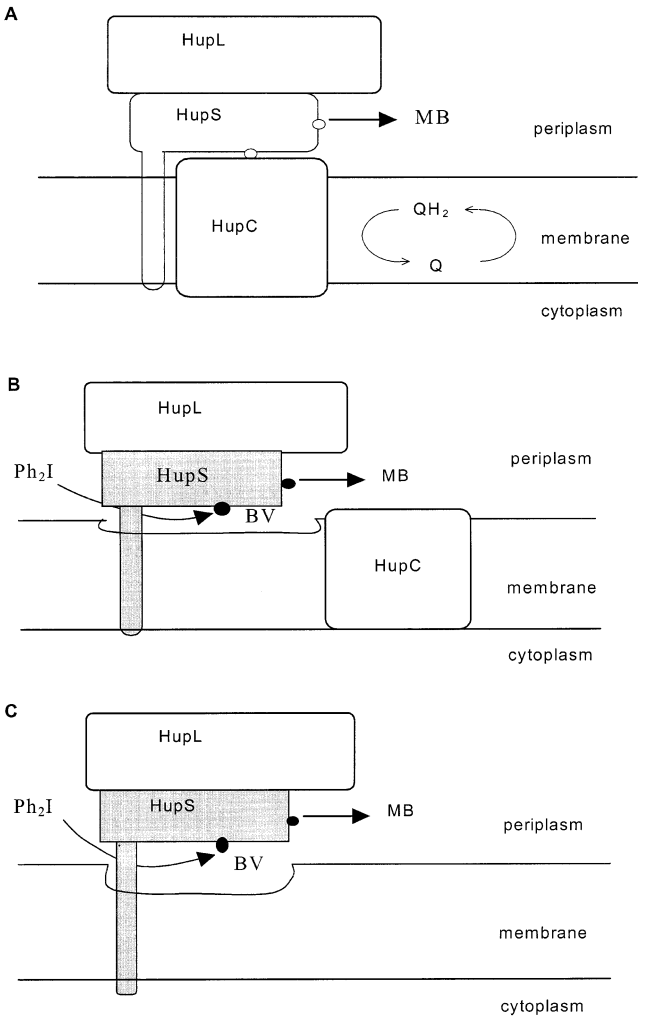

Fig. 7. Model proposed to explain *R. capsulatus* hydrogenase performances to various substrates in different conditions. (A) *R. capsulatus* whole cell strain B10. (B) Detergent extract of *R. capsulatus* whole cells strain B10. (C) *R. capsulatus* whole cells strain BCX1 HupC$^-$. The grey colour indicates a slight modification of the protein conformation induced by Ph_2I. BM: methylene blue. BV: benzyl viologen.

The presence of a methylene blue site on the hydrogenase complex could be only an experimental

curiosity without physiological significance. However, we cannot exclude the possibility that this high potential site play a physiological role. In this case, the hydrogenase will reduce in vivo an unidentified high potential periplasmic electron carrier. This carrier would later provide reducing power to one of the oxygen terminal oxidases present in the *R. capsulatus* membrane. This system would give to *R. capsulatus* an ultra short respiratory chain from H_2 to 0_2 with a low or null ATP yield but a high oxygen scavenger capacity. Alternatively, the use of the low potential site would result in a more traditional respiratory chain with a much higher ATP yield.

On an ecological point of view, the short respiratory chain would be well-adapted during the day (solar illumination) and under nitrogen fixing conditions. Under these conditions, the cyclic photosynthetic apparatus supplies large amounts of ATP to the nitrogenase and the hydrogenase system acts mainly as an oxygen scavenger to protect the nitrogenase. The hypothetical short respiratory chain would present the advantage to decouple the oxygen consumption of the ATP production.

On the other hand, during the night, without nitrogen fixation, the high yield respiratory chain would be used to extract as much energy as possible from the weak flux of hydrogen gas provided by the fermentative bacteria.

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