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Evidence for chemiosmotic coupling of reductive dechlorination and ATP synthesis in *Desulfomonile tiedjei*

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Abstract. *Desulfomonile tiedjei* (strain DCB-1) was previously shown to conserve energy for growth from reductive dechlorination of 3-chlorobenzoate coupled to formate oxidation. We tested the hypothesis that a chemiosmotic mechanism couples reductive dechlorination and ATP synthesis in *D. tiedjei*. Dechlorination resulted in an increase in the ATP pool of cells. Uncouplers and ionophores decreased both the dechlorination rate and the ATP pool. However, at low concentrations the inhibitors had relatively greater effects on the ATP pool, and in some cases, even appeared to stimulate dechlorination. Those agents could not completely inhibit ATP synthesis while allowing dechlorination activity. The proton-driven ATPase inhibitor, N,N′-dicyclohexylcarbodiimide (DCCD), had similar effects. An imposed pH gradient also resulted in an increase in the ATP pool of cells, and this increase was partially inhibited by DCCD. Addition of 3-chlorobenzoate to cell suspensions caused proton translocation by the cells. Proton translocation was stimulated by the permeant thiocyanate anion and inhibited by uncouplers. A maximum H^+/3-chlorobenzoate ratio of greater than two was observed. These findings suggest that dechlorination supports formation of a proton-motive force which in turn supports ATP synthesis via a proton-driven ATPase.

Key words: Anaerobic respiration – *Desulfomonile tiedjei* – Proton-driven ATPase – Proton-motive force – Proton translocation – Reductive dehalogenation – Respiratory inhibitors – Strain DCB-1 – Sulfate-reducing bacterium

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Abbreviations: 3CB, 3-chlorobenzoate; CCCP, *m*-chlorophenylhydrazone; DCCD, N,N′-dicyclohexylcarbodiimide; DNP, 2,4-dinitrophenol; ΔP, proton-motive force; PCP, pentachlorophenol

Desulfomonile tiedjei (strain DCB-1) is a sulfate-reducing bacterium (DeWeerd et al. 1990; Mohn and Tiedje 1990a) which is capable of the reductive dehalogenation of halobenzoates and chloroethenes (DeWeerd et al. 1986; Fathepure et al. 1987; Shelton and Tiedje 1984). This organism was shown to conserve energy for growth from reductive dechlorination of 3-chlorobenzoate (3CB) and 3,5-dichlorobenzoate (Dolfing 1990; Mohn and Tiedje 1990b). The addition of 3CB also supported ATP production in stationary phase cultures which had been limited for 3CB (Dolfing 1990). In growing cultures and in cell suspensions, reductive dechlorination of 3CB was shown to be coupled to formate oxidation (Mohn and Tiedje 1990b):

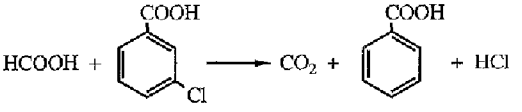

Fig. 1. Reductive dechlorination of 3CB coupled to formate oxidation

In addition, dechlorination probably can be coupled to H_2 oxidation (DeWeerd et al. 1991; Mohn and Tiedje 1990b). Subsequent investigation of medium requirements has allowed growth of *D. tiedjei* during serial passages on defined medium with formate plus 3CB as sole energy substrates (WW Mohn and JR Cole, unpublished). Dechlorination activity in *D. tiedjei* appears to be specifically induced by 3CB and certain of its analogues (Cole and Tiedje 1990).

Since the electron donors which support growth with 3CB, formate and H_2, are not known to support substrate-level phosphorylation, it seems likely that a chemiosmotic mechanism might couple dechlorination and ATP synthesis. Such a process would be a novel mode of anaerobic respiration and would be significant to basic understanding of energy metabolism as well as to applications of reductive dehalogenation for bioremediation. Respiratory inhibitors originally used in mitochondrial

systems have been successfully used to study anaerobic chemiosmotic mechanisms in a phylogenetically diverse range of bacteria including methanogenic bacteria (reviewed by Daniels et al. 1984), sulfate-reducing bacteria (Barton et al. 1970; Kramer and Cypionka 1989; Steenkamp and Peck 1981), acetogenic bacteria (Ivey and Ljungdahl 1986), dissimilatory iron reducing bacteria (Arnold et al. 1986) and fermentative bacteria (Cox and Henick-Kling 1989; Russel and Strobel 1989). The following inhibitors have been found broadly effective in various of the above studies and were employed in this study: the uncouplers, pentachlorophenol (PCP), 2,4-dinitrophenol (DNP), and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP); the ionophores, monensin and gramicidin; and the proton-driven ATPase inhibitor, N,N′-dicyclohexylcarbodiimide (DCCD). Proton translocation has been directly measured with a number of bacteria capable of various types of anaerobic respiration, including the reduction of sulfite, thiosulfate, nitrate, nitrite, nitrous oxide, fumarate, iron(III), mangenese(IV) and trimethylamine N-oxide (Barton et al. 1983; Boogerd et al. 1981; Fitz and Cypionka 1989; Garland et al. 1975; Kobayashi et al. 1982; Miki and Wilson 1978; Myers and Nealson 1990; Short and Blakemore 1986; Steenkamp and Peck 1981; Takagi et al. 1981).

The goal of this study was to test the hypothesis that a chemiosmotic mechanism couples reductive dechlorination and ATP synthesis by *D. tiedjei*. Using cell suspensions, we examined the effects of respiratory inhibitors on dechlorination of 3CB and on dechlorination-dependent ATP synthesis. The ability of an imposed pH gradient to support ATP synthesis was tested. Additionally, we tested whether dechlorination could create a proton-motive force by measuring 3CB-dependent proton translocation. The findings were uniformly consistent with the hypothesis of a chemiosmotic mechanism.

Materials and methods

Media

Desulfomonile tiedjei (strain DCB-1) was originally isolated in this laboratory (Shelton and Tiedje 1984). Stock cultures were grown on the mineral medium used for isolation with the following modifications and additions: the reductant was changed to 1 mM cysteine plus 0.1 mM titanium citrate, the vitamin solution was changed, and the following were added, 10 mM HEPES buffer (hemisodium salt), 20 mM sodium pyruvate and 1 mM sodium 3CB. Titanium citrate was prepared according to Zehnder and Wuhrmann (1976) and added from filter-sterilized stock solution. The vitamin solution was 500 µg/l nicotinamide, 200 µg/l 1,4-naphthoquinone, 50 µg/l thiamine and 50 µg/l lipoic acid (DeWeerd et al. 1990) added from 1000-fold concetrated, filter-sterilized stock solution with NaOH added to facilitate dissolution. Pyruvate was added from filter-sterilized stock solution. The gas phase was N_2-CO_2 (95:5), and the pH was adjusted to 7.5 before autoclaving. When depleted, 3CB was replenished from filter-sterilized stock solution. Inocula were 10%, and cultures were incubated at 37° C.

ATP synthesis and dechlorination experiments

Cells in log phase and actively dechlorinating 3CB were washed and concentrated (final protein concentration 90 to 186 µg/l) by the following method. Cells were harvested by centrifugation in the culture vessels (160-ml serum bottles) for 60 min at 750 × *g* at 4° C. The pellet was washed at 4° C in anaerobic buffer containing, 20 mM HEPES (except as otherwise noted), 10 mM sodium formate, 1 mg/l resazurin, 1 mM Na_2S and 0.5 mM titanium citrate, pH 7.5 under a gas phase of N_2. Cells were centrifuged again for 30 min and suspended in the above buffer. The headspace was N_2. Cell suspensions were maintained at 37° C and starved by incubation for 4 h in the absence of an electron acceptor (formate was present as an electron donor). Sodium 3CB was added from a neutralized stock solution (pH 7.5). When used, inhibitors were added from ethanol stock solutions 20 min prior to 3CB additions, and all treatments, including controls, received the same total amount (0.5%) of ethanol. HCl was added from a 5 M stock solution.

Proton translocation experiments

Cells in log phase and actively dechlorinating 3CB were concentrated (final protein concentration 0.32 – 1.07 µg/ml) by the following method of Patel (1984). Cells were drawn from the culture vessel onto a sterile filter cartridge (0.2 µm, Sartorius, Göttingen, FRG). Cells were washed by drawing 3 ml unbuffered, anaerobic solution (10 mM KCl or 10 mM NaCl) through the filter. Cells were suspended by expelling them from the filter with 4 to 5 ml of the same unbuffered, anaerobic solution into a reaction vessel described by Patel and Agnew (1981). The headspace of the vessel was constantly flushed with H_2 or N_2 using an anaerobic gassing apparatus. Cell suspensions were magnetically stirred and maintained at 35° C. The pH of cell suspensions was monitored with a combination pH/reference probe (Baxter, Toronto, Canada) coupled to a pH meter (New Brunswick Scientific, Edison, N. J., USA, model pH-40) and a chart recorder (Cole Parmer Instruments, Chicago, Ill., USA, model 8373-20). The pH monitoring system was calibrated by injection of a standard volumetric HCl solution (Anachemia, Montreal, Canada) into the cell suspension. The pH of cell suspensions was initially 6.8 – 7.0 and rising. After 3 – 4 h the pH stabilized at 7.5 – 8.0 and the experiments were begun. All additions to cell suspensions were by injection of neutralized anaerobic solutions. All results reported below were consistently observed in independent experiments.

Analyses

Benzoates were analyzed by high pressure liquid chromatography as described previously (Stevens et al. 1988) except the eluent consisted of water-acetonitrile-phosphoric acid (66:33:0.1) and the UV detector was set at 230 nm. ATP was extracted as follows: a 0.20-ml sample was added to 0.80 ml 95° C 20 mM Tris-HCl pH 7.8, incubated 5 min at that temperature and filtered (0.45 µm). ATP was assayed with luciferin-luciferase reagent in Tris-aspartate buffer (Sigma Chemical Co., St. Louis, MO) using a Chem-Glo photometer. A linear standard curve was obtained over the range of ATP concentrations assayed. Protein concentration was determined by a modification of the Lowry assay (Hanson and Phillips 1981).

Results

Dechlorination-dependent ATP synthesis

Upon addition of 3CB to cell suspensions, dechlorination immediately began and the cellular ATP pool rapidly

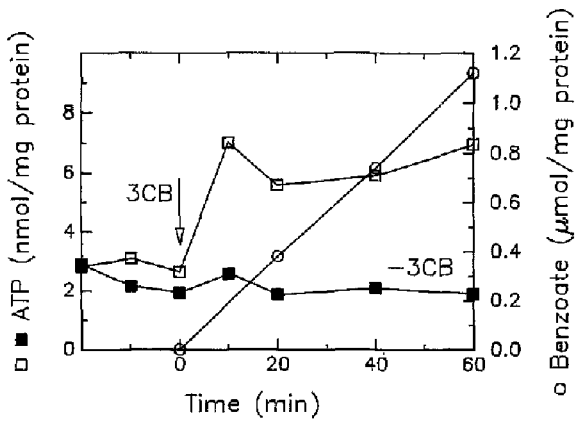

Fig. 2. Reductive dechlorination and consequent increase in the ATP pool of suspended cells. Buffer contained 20 mM Tris-HCl instead of HEPES. *Symbols*: ○ and □, suspension to which 1 mM 3CB was added at 0 min; ■, control to which no 3CB was added

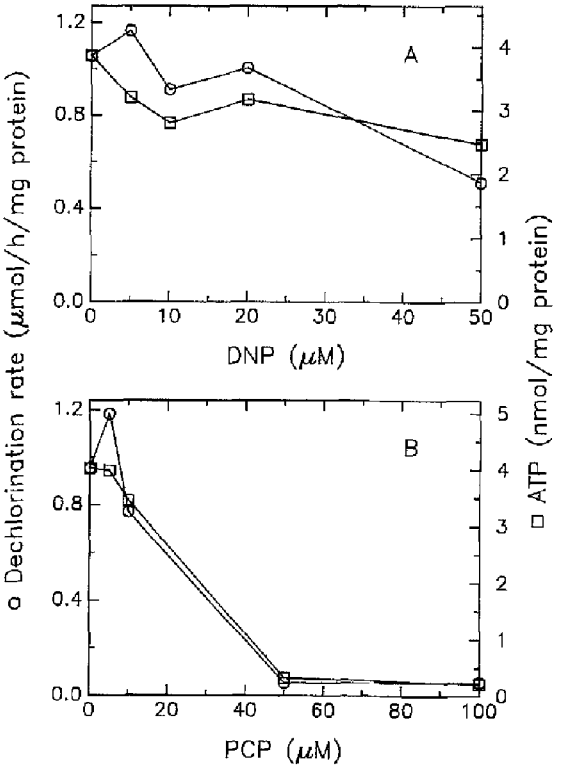

Fig. 3. Effect of the uncouplers, DNP (**A**) and PCP (**B**), on the dechlorination rate and ATP pool of suspended cells ($n = 3$ and 2, respectively)

increased approximately 3-fold (Fig. 2). Benzoate accumulated stoichiometrically as a product of 3CB. The dechlorination rate and the ATP pool remained constant for at least 1 h. In subsequent experiments examining the effects of respiratory inhibitors, benzoate and ATP were assayed 1 h after addition of 3CB to similar suspensions in order to estimate the dechlorination rate and ATP pool. In each experiment, controls without inhibitors or 3CB additions confirmed the dechlorination-dependent ATP pool increase.

Table 1. Effects of respiratory inhibitors at minimum effective concentrations on the dechlorination rate and ATP pool of suspended cells[a]

Treatment	Concentration (μM)	Relative dechlorination rate (%)[b]	Relative ATP pool (%)[b]	Ratio of ATP/dechlorination rate
Control	–	100 ± 1	100 ± 1	1.00
No 3CB	–	0	28 ± 1	–
DNP	5	109 ± 5	82 ± 8	0.75*
PCP	10	89 ± 10	69 ± 6	0.78**
Gramicidin	100	119 ± 8	94 ± 6	0.79**
Monensin	100	80 ± 8	69 ± 13	0.86*
DCCD	50	127 ± 2	69 ± 5	0.54**

[a] Except for that of DNP, data are from experiments independent from those shown in Figs. 3 and 4

[b] Data are means of triplicates ± standard error, normalized to value of parallel controls

* $P < 0.05$; ** $P < 0.01$, probabilities that ratios are not significantly lower than that of control by Student's *t*-test

Effects of respiratory inhibitors

The uncouplers, PCP and DNP, decreased both the dechlorination rate and the ATP pool of suspended cells (Fig. 3, Table 1). However, at relatively low concentrations, which caused only partial effects, PCP and DNP appeared to slightly stimulate dechlorination while decreasing or not changing the ATP pool. The uncoupler, CCCP, completely inhibited both dechlorination and the dechlorination-dependent ATP pool increase at the lowest concentration tested, 5 μM. The ionophore, gramicidin, stimulated dechlorination over the concentration range tested while decreasing the ATP pool at the higher concentrations (Fig. 4A, Table 1). Another ionophore, monensin, was tested only at 100 μM, and it decreased both the dechlorination rate and, to a relatively greater extent, the ATP pool (Table 1). At relatively low concentrations, both uncouplers and ionophores had the general effect of significantly decreasing the ATP pool relative to the dechlorination rate (Table 1).

The proton-driven ATPase inhibitor, DCCD, had effects similar to uncouplers and ionophores (Fig. 4B). At relatively high concentrations, DCCD decreased both the dechlorination rate and the ATP pool of suspended cells, but 50 μM DCCD increased the dechlorination rate while decreasing the ATP pool (Table 1).

Effect of imposed pH gradient

An imposed pH gradient from HCl addition caused an immediate increase, by approximately 50%, in the ATP pool of suspended cells (Fig. 5). Treatment of the cells with DCCD reduced both the initial ATP pool and the ATP pool increase due to imposition of a pH gradient.

3CB-dependent proton translocation

Addition of a neutral solution of 3CB to cell suspensions with an electron donor caused a rapid decrease in the

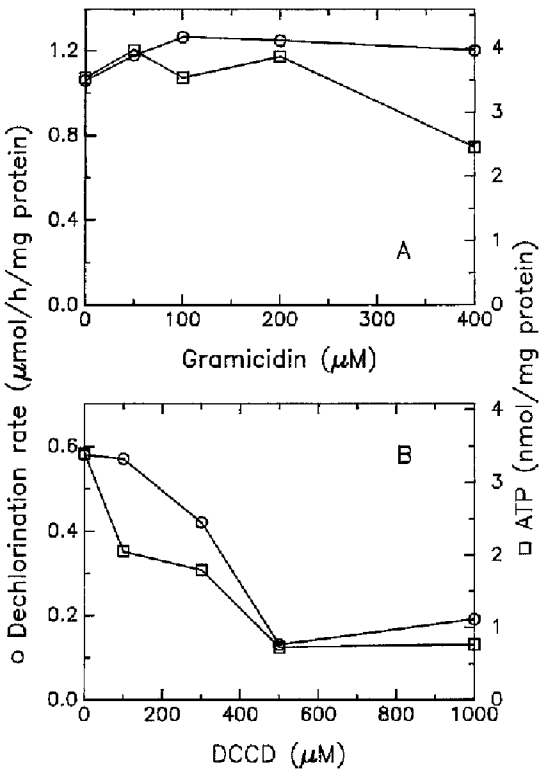

Fig. 4. Effect of the ionophore, gramicidin (**A**), and the proton-driven ATPase inhibitor, DCCD (**B**) on the dechlorination rate and ATP pool of suspended cells ($n = 3$ and **1**, respectively). For **B**, buffer contained 20 mM Tris-HCl instead of HEPES

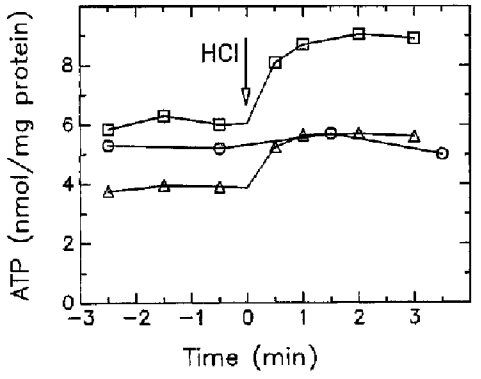

Fig. 5. Increase in the ATP pool of suspended cells resulting from an imposed pH gradient. *Symbols*: ○, control with no HCl addition; □, HCl added at 0 min reducing pH from 7.5 to 2.1; △, 200 μM DCCD added at −20 min and HCl added at 0 min reducing pH from 7.5 to 2.1 ($n = 2$)

medium pH (Fig. 6A). Either H_2 or formate could serve as the electron donor. In the absence of an electron donor, the pH response was slower and smaller and was not reproducible. With formate the pH baseline was less stable than with H_2, and addition of 25 μg/ml carbonic anhydrase did not stabilize the baseline. Therefore, H_2 was subsequently used as the electron donor. The pH response was observed both by addition of sodium 3CB to cells suspended in sodium chloride and by addition of potassium 3CB to cells suspended in potassium chloride.

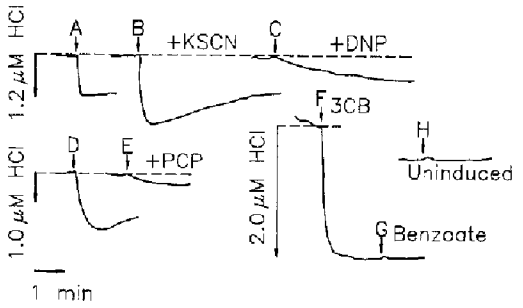

Fig. 6. 3CB-dependent proton translocation in cell suspensions shown by pH tracings. **A–C**: 1.2 μM 3CB additions, initially (**A**), after 20 mM KSCN addition (**B**) after KSCN and 40 μM DNP additions (**C**); protein, 1.1 mg/ml. **D, E**: 1.0 μM 3CB additions in presence of 20 mM KSCN, initially (**D**) and after 30 μM PCP addition (**E**); protein, 1.1 mg/ml. **F, G**: 2.0 μM additions of 3CB (**F**) and benzoate (**G**); protein, 0.48 mg/ml. **H**: 1.0 μM 3CB addition to uninduced cells

Table 2. Proton translocation by suspended cells upon addition of 1 to 2 μM 3CB

Treatment[a]	Mean H^+/3CB	Standard deviation	Replicates
No additions	1.2	0.05	6
KSCN	2.1*	0.43	12
KSCN + DNP	0.4*	0.00	2
KSCN + PCP	0.8*	0.00	2

[a] Concentrations = KSCN, 10–30 mM; DNP, 30 μM; PCP, 40 μM

* $P < 0.001$, probability that ratios are not significantly different than that of treatment with no additions by Student's t-test

Thus, neither sodium nor potassium appears to be required in the medium.

The magnitude of the above pH decrease relative to 3CB added (H^+/3CB ratio) was approximately 1 (Table 2), and following the decrease, the pH was relatively stable (Fig. 6A). However, preincubation of cells with potassium thiocyanate changed the pH response to 3CB, causing a greater pH decrease followed by a gradual increase (Fig. 6B). The maximum H^+/3CB ratio of 2.4 was observed with 20 mM potassium thiocyanate. Preincubation of cells with the uncouplers, DNP and PCP, greatly slowed the pH decrease in response to 3CB addition (Fig. 6B–E) and decreased the H^+/3CB ratio (Table 2).

The pH response was observed following 3CB additions in the range of 0.25–50.0 μM, with maximum proton translocation between 0.5 and 2.0 μM 3CB. Addition of sodium benzoate to cell suspensions caused no pH response (Fig. 6F, G). Addition of 3CB to cells which were grown in the absence of 3CB also caused no pH response (Fig. 6H). The latter two negative controls indicate that there was no direct effect on medium pH from either additions of stock solutions or from the benzoate product of dechlorination, and that 3CB-dependent medium acidification required cells induced for dechlorination activity.

Discussion

The ATP pool increase in suspended cells in response to addition of 3CB (Fig. 2) agrees with previous findings of Dolfing (1990) using stationary phase cultures and indicates that dechlorination and ATP synthesis are coupled. With the exception of CCCP, all uncouplers and ionophores tested consistently had the effect of significantly reducing the ATP pool relative to the dechlorination rate (Table 1), thus, apparently reducing the efficiency of dechlorination-dependent ATP synthesis. Uncouplers and ionophores are both able to dissipate a proton-motive force (ΔP). The similar effect of such a variety of agents suggests that the mode of action was dissipation of a ΔP rather than an artifact of unexpected activities. On the other hand, the incongruous effect of CCCP might be such an artifact. Structurally CCCP resembles 3CB, being an aromatic compound with a chlorine substituent *meta* to an alkyl substituent, and it is plausible that the extreme sensitivity of dechlorination to CCCP could be due to its direct inhibition of the dechlorinating enzyme.

The apparent reduction in the efficiency of dechlorination-dependent ATP synthesis due to dissipation of ΔP (Table 1) is consistent with chemiosmotic coupling of dechlorination and ATP synthesis. In such a system formate oxidation or H_2 oxidation coupled to 3CB reductive dechlorination may support formation of a ΔP which, in turn, may support ATP synthesis via a proton-driven ATPase. However, these results alone do not rule out the alternative hypothesis that dechlorination supports substrate-level phosphorylation which, in turn, supports formation of a ΔP via a proton-driven ATPase (performing the reverse function as that above). Also, while these results suggest the involvement of H^+ as a coupling ion, they do not rule out the additional significance of other ions.

Relatively low concentrations of DNP, PCP and gramicidin appeared to stimulate dechlorination (Figs. 3 and 4A, Table 1). This effect resembles the observation in mitochondrial systems that oxygen consumption is stimulated by uncouplers. Similar observations have also been made by Blaut and Gottschalk (1984) studying methogenesis from H_2 plus methanol by *Methanosarcina barkeri* and by Hansen et al. (1988) studying caffeate reduction by *Acetobacterium woodii*. A possible explanation for this effect on *Desulfomonile tiedjei* is consistent with the hypothesis that dechlorination supports formation of a ΔP. In this case, the free energy of the dechlorination reaction might be related to the ΔP, and the uncouplers and ionophore, by dissipating the ΔP, might make dechlorination thermodynamically more favorable. The stimulation of dechlorination by these agents is thus consistent with chemiosmotic coupling of dechlorination and ATP production.

The finding that low concentrations of DCCD do not inhibit dechlorination while they do reduce the ATP pool (Fig. 4B; Table 1) suggests that the action of DCCD is specific to a proton-driven ATPase which is involved in converting ΔP to ATP. The existence of such an ATPase in *D. tiedjei* is further supported by the finding that an imposed pH gradient (inside alkaline) causes an increase in the ATP pool (Fig. 5). As expected, this ATP increase also appears to be sensitive to DCCD; although, inhibition was not complete with the concentration of DCCD used. These results are also consistent with chemiosmotic coupling of dechlorination and ATP synthesis, but not with the alternative hypothesis above. If an ATPase was functioning to translocate protons rather than to produce ATP, then one would not expect DCCD to decrease the ATP pool. The stimulatory effect of a low concentration of DCCD on dechlorination (Table 1) is difficult to explain, and we can only speculate that it might involve a response to the accompanying decrease in the ATP pool. This observation does not appear to favor either a chemiosmotic mechanism or the alternative hypothesis.

The inability of uncouplers, ionophores and DCCD to completely inhibit ATP synthesis while allowing dechlorination (i.e., to completely uncouple ATP synthesis and dechlorination) may indicate that energy, in the form of either ATP or a ΔP, is required for dechlorination (although, there is a net energy gain from the overall process). Possible explanations would be that dechlorination requires active transport or an activation step. The requirement of an activation step in sulfate reduction is well known, and such a step was also proposed for methanogenesis by Mountfort (1978). A requirement for active transport of sulfoxy anions by *Desulfovibrio desulfuricans* was indicated in studies by Cypionka (1987).

The observation of 3CB-dependent proton translocation (Fig. 6) indicates that dechlorination can generate a ΔP and is also consistent with chemiosmotic coupling of dechlorination and ATP synthesis. The H^+/3CB ratio of 1 initially observed (Fig. 6A, Table 2) does not prove that proton-translocation occurred, since this corresponds to the substrate/product ratio of the two species in the reductive dechlorination reaction. However, the increased H^+/3CB ratio of 2 resulting from addition of the permeant thiocyanate anion (Fig. 6B, Table 2) does indicate bona fide proton translocation. This effect of thiocyanate is consistent with that observed in other studies (Fitz and Cypionka 1989; Barton et al. 1983; Steenkamp and Peck 1981). This effect has been explained by the ability of the permeant thiocyanate anion to dissipate membrane potential which is increased by proton translocation and which makes further proton translocation less favorable. The observed inhibition of proton translocation by uncouplers (Fig. 6C, E, Table 2) is also expected and has been observed in other studies (Barton et al. 1983; Fitz and Cypionka 1989, 1991; Kobayashi et al. 1982; Myers and Nealson 1990; Short and Blakemore 1986; Takagi et al. 1981). This effect of uncouplers is further evidence that the 3CB-dependent pH response observed involves formation of a ΔP and is not simply the formation of protons as a product of the reductive dechlorination reaction.

The above results provide a consistent body of evidence supporting chemiosmotic coupling of reductive dechlorination and ATP synthesis in *D. tiedjei*. The general agreement of the above results is critical, as the methods used provide only indirect evidence. Lancaster (1986) has even argued that results with methanogens

similar to those reported here with *D. tiedjei* are not inconsistent with ATP synthesis via substrate-level phosphorylation. Further understanding of the mechanism involved in conservation of energy from reductive dechlorination might come from identification of electron carriers involved, localization of the enzymes involved relative to the cell membrane or development of an in vitro system capable of dechlorination-dependent ATP synthesis.

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