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Proton translocation coupled to formate oxidation in anaerobically grown fermenting *Escherichia coli*

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Abstract

Proton translocation, coupled to formate oxidation and hydrogen evolution, was studied in anaerobically grown fermenting *Escherichia coli* JW136 carrying hydrogenase 1 (*hya*) and hydrogenase 2 (*hyb*) double deletions. Rapid acidification of the medium by EDTA-treated anaerobic suspension of the whole cells or its alkalization by inverted membranes was observed in response to application of formate. The formate-dependent proton translocation and $2H^+$–K^+ exchange coupled to H_2 evolution were sensitive to the uncoupler, carbonylcyanide-*m*-chlorophenylhydrazone, and to copper ions, inhibitors of hydrogenases. No pH changes were observed in a suspension of formate-pulsed aerobically grown ("respiring") cells. The apparent H^+/formate ratio of 1.3 was obtained in cells oxidizing formate. The $2H^+$–K^+ exchange of the ATP synthase inhibitor *N*,*N*′-dicyclohexylcarbodiimide-sensitive ion fluxes does take place in JW136 cell suspension.

Hydrogen formation from formate by cell suspensions of *E. coli* JW136 resulted in the formation of a membrane potential ($\Delta\psi$) across the cytoplasmic membrane of −130 mV (inside negative). This was abolished in the presence of copper ions, although they had little effect on the value of $\Delta\psi$ generated by *E. coli* under respiration. We conclude that the hydrogen production by hydrogenase 3 is coupled to formate-dependent proton pumping that regulates $2H^+$–K^+ exchange in fermenting bacteria.

Keywords: Formate hydrogen lyase; [NiFe] Hyc hydrogenase; Ion exchange-coupled proton translocation; Fermentation; *Escherichia coli*

1. Introduction

Escherichia coli catalyses cleavage of formate to H_2 and CO_2 using the membrane-associated enzyme complex formate hydrogen lyase (FHL) [1,2]. FHL is only produced under anaerobic conditions and in the absence of alternative terminal electron acceptors, such as nitrate. This pathway is active at low pH and high formate concentrations, and is thought to provide a deacidification system countering the build-up of formate during fermentation. FHL includes a formate dehydrogenase isoenzyme (FDH-H; *fdhF*), a third hydrogenase isoenzyme (Hyc, *hyc*), or a fourth (Hyf, *hyf*) hydrogenase isoenzyme. Both hydrogenases form FHL-1 and FHL-2 pathways that catalyze hydrogen production when the cells are grown at a starting pH of 7.5 or 6.5, respectively [3]. The *hyc* operon, in addition, encodes an electron transfer protein (HycB) with four predicted [4Fe–4S] clusters. It is assumed to function as an electron carrier between formate dehydrogenase and hydrogenase [4]. Hyc and Hyf hydrogenases catalyze the reduction of H^+. This exergonic process might be coupled to energy conservation by means of electron-transport phosphorylation. It has been recently reported [5–7] that the subunits of these enzymes, including the two integral membrane subunits, were found to be closely related to subunits of energy-conserving NADH:quinone oxidoreductase (Complex I). HycE and HycG in *E. coli* Hyc are related to the hydrogenase large and small subunits conserved in all [NiFe] hydrogenases

Abbreviations: FHL, formate hydrogen lyase; CCCP, carbonyl cyanide-*m*-chlorophenylhydrazone; DCCD, *N*,*N*′-dicyclohexylcarbodiimide; DTT, DL-dithiothreitol; BSA, bovine serum albumin; $\Delta\psi$, membrane potential; *hya*, *hyb*, *hyc*, *hyf*, hydrogenases 1–4 operons, respectively; EDTA, ethylenediaminetetraacetic acid; TPP^+, tetraphenylphosphonium bromide.

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and more closely related to subunits of the energy-conserving NADH:quinone oxidoreductase (Complex I) [8]. Primary candidates for harboring an ion-translocating machinery are three large membrane- spanning proteins in Complex I and one to three ditto proteins in Hyc and Hyf [9]. The closer resemblance of Hyc and Hyf to Complex I, as compared to standard [NiFe] hydrogenases, implies that the enzymes could function as ion pumps.

Our results also confirm this hypothesis. We have previously demonstrated (reviewed in Ref. [10]) that the FHL system couples along with the K^+ uptake by a low-affinity TrkA system: K^+ uptake was not present in a strain deleted for components of FHL. It also requires the F_0F_1–H^+-translocating ATP synthase: both H_2 production and H^+–K^+ exchange were lost in mutant strains lacking the F_0 sector of the ATP synthase. Therefore, like TrkA-mediated K^+ uptake, FHL activity is also F_0-dependent. Thus, the three activities, ATP-dependent H^+ pumping by the ATP synthase, K^+ transport by TrkA, and H_2 production by the FHL, had been functionally linked by carrying out the *N*,*N*′-dicyclohexylcarbodiimide (DCCD)-sensitive $2H^+$–K^+ exchange and H_2 evolution. The role of FHL activity in $2H^+$–K^+ exchange reaction is not yet understood. One possibility is that the FHL generates a proton motive force, which is necessary for the functioning of the TrkA system. Under standard substrate concentrations, the FHL reaction is not an exergonic process. However, at low H_2 partial pressures and low pH, in vivo conditions, the reaction becomes exergonic (~−20 kJ/mol) [7,11] (i.e., from a thermodynamic point of view, the reaction could be coupled to energy conservation).

Thus far, it has not been experimentally tested if the *E. coli* FHL reaction is coupled to energy conversation and the molecular mechanisms underlying this reaction are not understood. No proton pumping is detected yet although the operon encoding Hyf is suggested to have genes for proton-translocating components. The Hyc enzyme is extremely labile but much of which is known today about the synthesis of the [NiFe] center in hydrogenases was deduced from experiments with this enzyme [12]. Our electrochemical results presented in this paper indicate oxidation of formate by *E. coli* and reduction of protons by FHL, resulting in the formation of a proton gradient across the bacterial membranes (Fig. 1). The latter could be involved in the regulation of ATP-driven proton–potassium exchange.

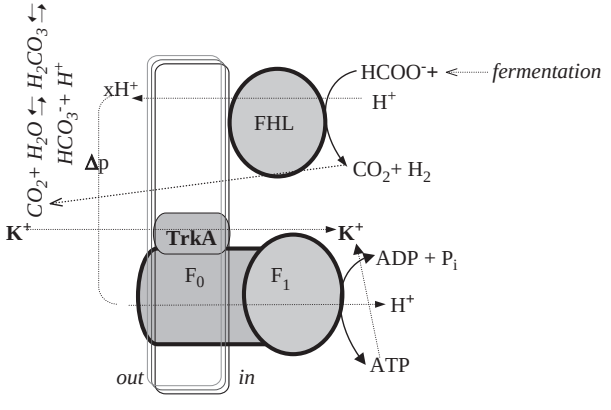

Fig. 1. Schematic representation of the coupling between the FHL and the ATPase in intact cells of *E. coli*. Formate is oxidized by the FHL, which reduces H^+ to produce H_2. As the primary proton-translocating complex, FHL also pumps H^+ across the membranes, resulting in a transmembrane proton gradient. This gradient can then be used by the secondary proton translocating complex, ATPsynthase, to produce ATP. ATP hydrolysis leads to a countergradient K^+ uptake.

2. Materials and methods

2.1. Bacterial strains and preparation of bacteria and washed vesicles

E. coli JW136 hydrogenase 1 (*hya*) and hydrogenase 2 (*hyb*) double deletion (*Δhya/Δhyb*) mutant (the genotype is Hfr (PO1 of Hfr Hayes), *Δ(gpt–lac)*5, *rel*A1, *spo*T1, *thi*-1, *Δhya*(km^R), *Δhyb*(km^R), and its precursor strain BW 545 were gifts from K.T. Shanmugam. *E. coli* HD 700 (*ΔhycA-H*) altered Hyc [13] and its precursor strain MC4100 were kindly provided by A. Böck, and used throughout. Bacteria were grown under anaerobic conditions at 37 °C in LB medium (pH 6.5) or under aerobic conditions that were detailed elsewhere [3,14]. 0.2% (wt/vol) glucose or 32 mM sodium succinate was added appropriately. Fermentative gas (H_2) evolution upon bacterial growth was confirmed by a chemical assay [15]. The pH of the growth medium decreased from 6.5 to 6.2 during the course of fermentative conditions; fermentation or respiration by *E. coli* under growth conditions used was described previously [3,14]. The growth was followed by measuring the optical density at 600 nm, and the cells were harvested by centrifugation and washed twice. Preparation of whole cells and inverted vesicles has been done as described previously [16,17].

Bacto-agar, tryptone, and yeast extract were from Roth (Germany); DL-dithiothreitol, DTT, tetraphenylphosphonium (TPP^+) bromide, potassium thiocyanide, formate (sodium salt), acetic acid, DCCD, $CuCl_2$, and CCCP were from Sigma (USA).

2.2. Proton translocation studies

Proton translocation has been monitored at 37 °C as was presented previously [16,18,19]. Inverted membrane vesicles were prepared as described [16,20] using Tris–Cl buffer instead of potassium phosphate one. 80–100 μl of inverted vesicles (2.5 mg of protein/assay) or 50–80 μl of the whole cells (0.6–0.8 mg/ml protein) pre-treated with 10 mM EDTA was introduced into the medium that contained 40 mM potassium thiocyanide solution with 0.5 M sucrose, 1 mg/l resazurine, and 10 mM DTT. Different concentrations of sodium formate, lactate, CCCP, or DCCD were added and the related pH changes were measured. In control

experiments, the response after ATP (disodium salt) addition was assayed. The mixture was constantly stirred with a small magnetic stir bar. Additions were made with microliter syringes, which were previously degassed by using anaerobic buffer. The pH value of the stock solutions used in the various assays was adjusted to about 6.2–6.3 (the rough pH of the experimental medium). Changes in pH were calibrated by nanomolar addition of an anaerobically prepared HCl or NaOH solution. After calibration of the system using nanomolar amounts of standard HCl solutions, the ratio between protons and formate was calculated as the difference of the maximal degree of acidification and the baseline.

2.3. Determination of membrane potential

Generation of membrane potential ($\Delta\psi$) was determined with TPP^+-selective electrode in cells treated with 10 mM EDTA, as described previously [21]. The value of $\Delta\psi$ was calculated by distribution of TPP^+ between the cytoplasm and the medium. 1 μM TPP^+ was introduced into the assay mixture, binding of TPP^+ on the bacterial surface was determined after boiling for 3–5 min, and the cytoplasmic value of 0.86×10^{-9} μl/cell was employed [21].

2.4. K^+ and H^+ transport assays

The transfer of ions across the bacterial membranes was assessed from the variation of their activity in the medium. K^+ and H^+ fluxes through the bacterial membrane in whole cells were measured using selective electrodes as described elsewhere [3,14,15]. The assay mixture contained: 200 mM Tris-phosphate, 0.4 mM $MgSO_4$, 1 mM NaCl, and 1 mM KCl; pH was the same as for the growth of bacteria. Ion fluxes are expressed as the change in external activity of this ion per minute per quantity of bacteria in cubic centimeters. Small changes in K^+ and H^+ activities were recorded using a potentiometer and calibrated by titration with 0.02 mM KCl and 0.1 mM HCl, respectively. The transfer of the bacteria from the medium with a low osmolarity to that with a high osmolarity was defined as a hyperosmotic stress, and the transfer back as a hypoosmotic stress [4]. The osmolarity of the solutions was increased by sucrose (non-metabolizing sugar) additions [15,22]. H_2 evolution was assayed as described in Ref. [15].

For DCCD inhibition studies, vesicles were incubated with 5 μM DCCD for 20 min at 37 °C. Protein concentration was measured by the Lowry method [23] using bovine serum albumin (type V; Serva) as standard. All assays were done at 37 °C. The average data are presented from two or three independent measurements; standard error did not exceed 5%. Each kinetic curve is based on the averaged data from three or five experiments of these series.

3. Results and discussion

3.1. Proton translocation by suspensions of E. coli JW136 whole cells

Anaerobically grown fermenting *E. coli* JW136 bacteria were tested for their ability to couple the conversion of formate to CO_2 and H_2 with the translocation of protons across the cytoplasmic membrane. The electrogenic translocation of protons is only measurable when the concomitant build-up of a transmembrane electrical gradient $\Delta\psi$ is prevented [24]. Therefore, concentrated cells were diluted with an unbuffered sucrose–thiocyanide solution and were pulsed with formate. Oxidation of formate by *E. coli* JW136 at pH 6.5 (when Hyc activity is expressed) [3,25] was associated with rapid liberation of protons into the medium (Fig. 2A) Acidification was followed by slow alkalization due to proton backflow into the cells. The extent of acidification was dependent on the amount of formate added (Fig. 2A). After calibrating the system by the addition of a standard solution of HCl, the extent of reversible acidification was calculated. An average H^+/formate ratio of approximately 0.8 was obtained, meaning that for every mole of formate oxidized to CO_2, 0.8 mol of protons were released to the medium.

Proton liberation was not completely reversible, finally approaching a net acidification of 0.5 formate/H^+. This acidification was demonstrated by stoichiometric titration

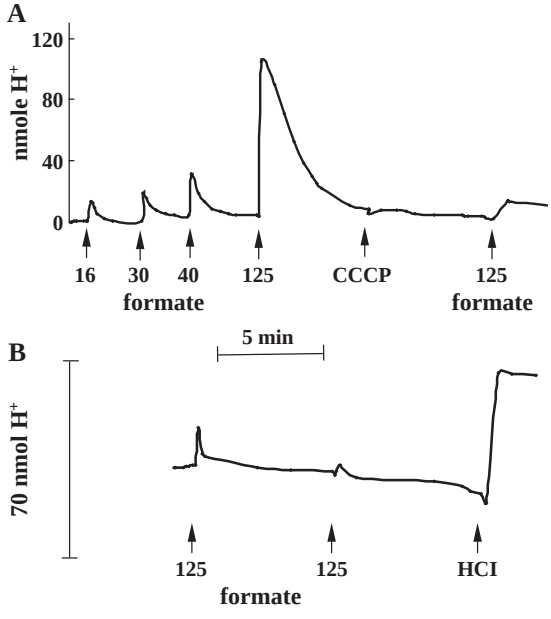

Fig. 2. Proton translocation (change in pH) coupled to the conversion of formate to CO_2 and H_2 by whole cell suspension of *E. coli* JW136 (A) and *E. coli* HD 700 (B) at pH 6.2–6.3. The reaction mixture was as described under Materials and methods section. Arrows indicate the moments of addition of formate (nmol). These additions resulted in a change of the pH value in the weakly buffered reaction mixture. CCCP was added as an ethanolic solution to a final concentration of 22 nmol/mg protein. Experiments were carried out at 37 °C.

with NaOH, restoring the initial pH. The influence of uncoupler on proton translocation and formate oxidation by cell suspensions was studied. In the presence of the protonophore CCCP, the transient acidification observed after addition of formate was strongly reduced (Fig. 2). In the presence of this proton-conducting agent, the membrane becomes specifically permeable to protons and can no longer sustain a proton potential. This further indicated that the observed acidification, upon formate addition, was mainly due to electrogenic proton translocation. In control experiments, formate was replaced with water or acetate, and no acidification was observed, indicating that H^+ transfer was specifically coupled with formate oxidation. The effect of ethanol on proton translocation by cell suspension was checked because ethanolic solution of uncoupler CCCP was used, and it was shown that ethanol did not affect H^+ translocation.

It was obtained in a separate experiment (data not shown) that the uncoupler did not alter the rate of H_2 formation (i.e., the addition of CCCP did not affect the rate of formate oxidation).

No acidification was observed after repeated pulses of formate when the reaction was performed in a suspension *E. coli* HD700 altered Hyc enzyme [13] (Fig. 2B). Anaerobically glucose-fermenting cells were grown under pH 6.5, which prevents an expression of FHL Hyf subunit.

3.2. Proton translocation by inverted vesicles

To elucidate the coupling between electron transport and ion translocation in another way, a subcellular system from *E. coli* JW136 that consisted of inverted membrane vesicles has been used. The addition of formate to this suspension led to a short period of alkalization of the suspension medium, which is thought to be due to a rapid proton movement from the medium into the lumen of the inverted vesicles. The alkalization was followed by a longer period of acidification until a stable baseline (pH) was reached again (Fig. 3). It is assumed that the consumption of the substrate (formate) is responsible for this effect. The energy-conserving electron transport comes to the end, causing a decay of the generated $\Delta\mu_H^+$ by passive diffusion of protons from the lumen of the inverted vesicles to the medium [19].

In an attempt to demonstrate that Hyc is responsible for formate-dependent proton translocation through the membranes and, hence, energy coupling in the system, the effect of the copper ions, a very specific and powerful inhibitor of the hydrogenases [26], was investigated (Fig. 3): the formate-dependent alkalization of the medium was completely lost after incubation of the vesicles with $CuCl_2$ (Fig. 3). An alkalization of the medium was successfully demonstrated under conditions when fermentation enzymes are suppressed [27]: aerobically grown *E. coli* JW136 cells couple ATP hydrolysis to protons translocation (Fig. 4). No transient alkalization was observed after pulse of formate followed after ATP injection (Fig. 4). These results allow

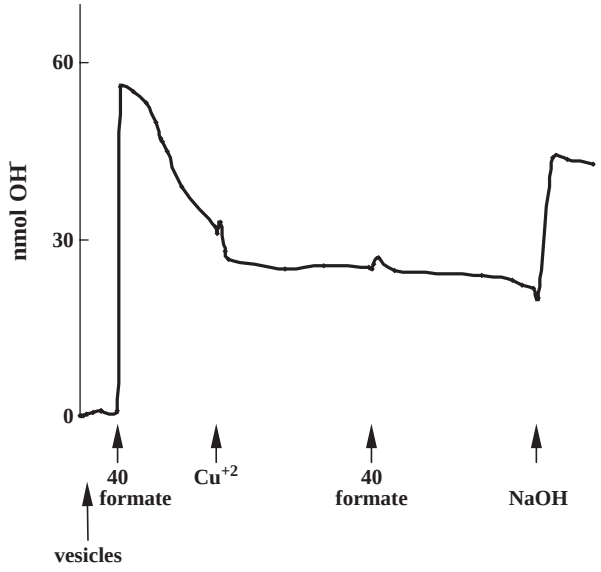

Fig. 3. Proton uptake coupled to the oxidation of formate by inverted membrane vesicles from *E. coli* JW136. Reactions were initiated by addition of nanomolar (40 nmol) amounts of formate. Where indicated, the following agents were added: $CuCl_2$, 250 nmol/mg protein; NaOH, 25 nmol/mg protein. The difference between the starting baseline and the final baseline was due to alkalization by the additions.

distinguishing between the ATP-dependent and formate-dependent alkalizations and strongly suggest that the FHL uses protons derived from formate as a means of conserving energy by pumping them across the *E. coli* membranes.

3.3. Ion exchange and membrane potential in E. coli mutants with defects in FHL

In these series of experiments, H^+ extrusion by H^+-translocating F_0F_1 ATPase and K^+ uptake through the TrkA system by anaerobically grown glucose-fermenting *E. coli* were studied. It was suggested that during anaerobic fermentative growth on glucose, but not in respiring cells [9,22], the H^+–K^+ exchange occurs with rigid stoichiometry (the exchange of $1K^+$ from the medium to $2H^+$ from a cytosol) of DCCD-sensitive fluxes. In the case of respiration, F_0F_1 and TrkA operate separately, directly utilizing a respiratory chain-generating $\Delta\mu_H^+$ (reviewed in Ref. [9]). We suggested that formate provided by *E. coli* under the mixed-acid fermentation of glucose could supply reducing equivalents required for a coupled operation of F_0F_1 and TrkA.

In Fig. 5A, kinetic curves of H^+–K^+ exchange by *E. coli* JW136 in response to introduction of formate are shown. The secondary osmotic stress induced by the addition of non-metabolizing sugar, sucrose [22], to the medium leads to the increase of H^+ extrusion and K^+ uptake. The stoichiometry of the DCCD-sensitive ion fluxes sensitive to DCCD is equal to $2H^+$ (it has to be considered that the lactic acid generated upon glucose fermentation is dissociated at slightly alkaline cytoplasmic pH and released to the external medium as $2H^+$ and $1Lac^-$ anion [28]) per $1K^+$

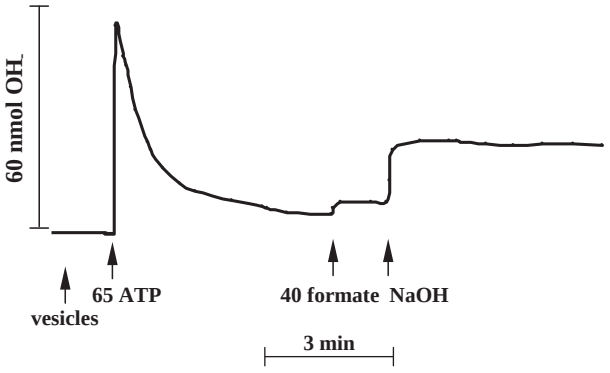

Fig. 4. Proton uptake coupled to the hydrolysis of ATP by inverted membrane vesicles from *E. coli* JW136 grown under aerobic conditions in the presence of sodium succinate. Proton translocation was not initiated by addition of formate. Experiments were carried out at 37 °C and pH 6.3. Arrows indicate an introduction of nanomolar amounts of reagents.

from the medium. The latter is stable upon variation of the medium pH and activity of K^+ in it. K^+ uptake is lost at a negative osmotic stress (data not shown). *E. coli* BW545 cells generate the $\Delta\psi$ of −132 mV, which was observed simultaneously upon addition of formate and was coupled to ion transport with fixed stoichiometry (Table 1).

The physiological role of FDH H as part of the FHL complex has to be considered. FDH H decomposes formic acid to H_2 and CO_2. The latter could also serve to create a proton gradient, as the protons of formic acid are removed from the cell in the form of hydrogen gas. The crystal

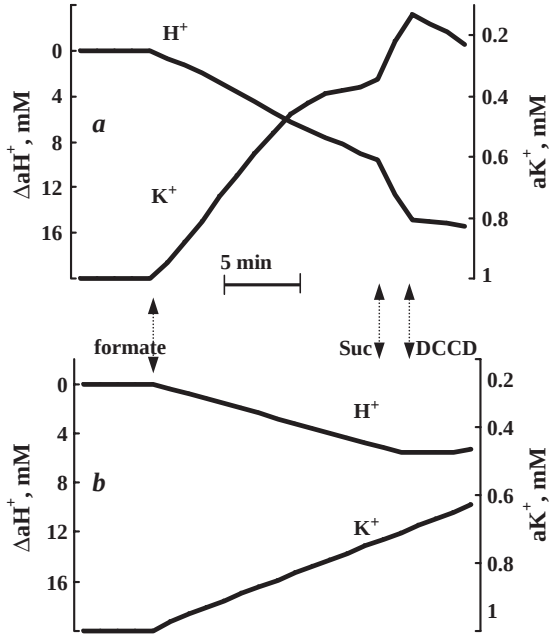

Fig. 5. Simultaneous recording of formate-depending H^+ and K^+ fluxes by anaerobically grown *E. coli* JW136 double *Δhya/Δhyb*$^-$ mutant strain (A) and *E. coliΔhycA-H* HD700 mutant (B). Positive osmotic stress was induced by addition of 440 mOsm (10%, wt/vol) of sucrose. Formate (22 mM) was added after the cells; the amount of DCCD was 0.1 mM. The kinetic curves given in the figures are one of three to five identical measurements.

Table 1
Stoichiometric characteristics of H^+–K^+ exchange and $\Delta\psi$ in *E. coli* precursor strains and mutants with defects in FHL

E. coli strains	Stoichiometry of H^+–K^+ exchange[a]	$\Delta\psi$[b] (mV)		
		No inhibitor	+DCCD	+$CuCl_2$
BW 545	2.0±0.1	−132	−121	−119
JW136	1.8±0.07	−130	−121	−97
MC4100	2.0±0.1	−137	−116	−118
HD 700	Unstable	−121	−117	−118

[a] Stoichiometry is calculated as a ratio of DCCD-sensitive initial fluxes upon a positive osmotic stress under different medium pH values and K^+ activity and represented with standard error.
[b] Average data.

structure of FDHs and related enzymes supports predictions that the enzymes are involved in proton motive force generation [29]. However, this is true for *E. coli* under conditions of respiration, when formate produced from pyruvate serves as a major donor of electrons to a variety of inducible respiratory pathways that use terminal acceptors other than molecular oxygen. In contrast to *E. coli* JW136 strain, the anaerobically grown glucose-fermenting *E. coli* HD700 *Δhyc* mutant carries out H^+–K^+ exchange upon addition of formate: it acidifies the medium, but does not uptake K^+ noticeably (Fig. 5B). While K^+ uptake is inhibited by DCCD that is typical for TrkA [30,31], it is insensitive to osmotic stress. The stoichiometry for DCCD-sensitive H^+ and K^+ fluxes is variable. The mutant generates an $\Delta\psi$ of −120 to −123 mV. In this case, it was necessary to distinguish whether H^+-translocating ATPase or Hyc is responsible for such a generation of $\Delta\psi$ in both (JW136 and HD 700) mutants. It is true indeed since ATPase itself possessed formate-dependent ATPase activity [17]. The effect of DCCD in a concentration that efficiently suppressed ATP-dependent H^+-translocating activity of F_0F_1 (data not shown) and inhibited ATPase activity [17] was negligible: $\Delta\psi$ decreased slightly (Table 1). This could also be demonstrated most effectively by the addition of copper ions. They abolish $\Delta\psi$ generation even though, at the same concentration, copper has no effect on the ATP-dependent generation of $\Delta\psi$ by *E. coli* inverted vesicles of precursor strains (Table 1).

The main explanation for data presented above may be proposed: two FHL pathways, FHL-1 and FHL-2, were suggested for *E. coli* carrying out a mixed-acid fermentation of glucose. FHL-1 mainly consisting of formate dehydrogenase H (Fdh-H) and Hyc [30] is active at acidic pH [32]. FHL-2 is mostly constituted by Fdh-H and Hyf [10], and appears to be responsible for formate oxidation to H_2 and CO_2 at alkaline pH [3]. Furthermore, proton translocation, coupled with oxidation of formate at pH 6.5 in appropriate mutants, can be related with the function of Hyc, operating under this pH value.

In the evaluation of H^+/formate stoichiometries, it has to be considered that formate is taken up via a proton symport-based mechanism [33] (i.e., 1 mol of protons is removed

from the external medium per mole of formate that is oxidized). It also has to be taken into account that CO_2, the product of formate oxidation, is a weak acid with an apparent pK_1 of 6.3. At pH 6.3, 50% of the H_2CO_3 is dissociated ($H_2CO_3 \leftrightarrow HCO_3^- + H^+$) (Fig. 1). At pH 6.3, 50% of the H_2CO_3 is dissociated; thus 0.5 mol of protons should be generated and released to the external medium per mole of formate oxidized. These both factors lead to a deficit of 0.5 mol of protons in the external medium; hence, this amount is to be added to the previously measured value, resulting in an overall H^+/formate stoichiometry of 1.3. If we take into account that under in vivo conditions the Gibbs energy change (ΔG) is about −20 kJ/mol [7,11], the proton motive force, Δp, can yield over 160 mV. This appears to be thermodynamically more than favorable for chemiosmotic ATP synthesis involving the FHL reaction.

The important feature of our findings is coincidence in time of $2H^+$–K^+ exchange activity, H_2 evolution, and changes of the value of $\Delta\psi$. It is worth noting that the value of $\Delta\psi$ in *Δhya/Δhyb* mutant (JW136) remained sufficiently high (Table 1) while in Δ*hyc*$^-$ mutant (HD 700), the noticeable drop in $\Delta\psi$ value is observed. Elimination of $\Delta\psi$ by copper ions resulted in suppression of Hyc and $2H^+$–K^+ exchange in anaerobically grown fermenting *E. coli*, clearly demonstrating the role of Hyc in $\Delta\psi$ generation.

A proton-pumping function is also assumed for Ech hydrogenase from *Methanosarcina barkeri* [7]. Cell suspension experiments have shown that the conversion of CO to CO_2 and H_2 is coupled to the translocation across the cytoplasmic membrane of approximately two protons per molecule of CO oxidized [18]. An interesting observation that has to be discussed in this context is that mutants of *E. coli* and *Salmonella typhimurium*, which are not able to synthesize an active F_0F_1-ATPase, do not produce H_2 when cultivated under fermenting conditions [34]. Hence, a functional ATPase seems to be required for the FHL reaction. There are two possible explanations for this effect.

In fermenting cells under anaerobic conditions, F_0F_1-ATPase might be an essential part of the H^+ efflux system associated with the FHL reaction as previously proposed [29]. Alternatively, if the FHL reaction were coupled to the generation of a proton motive force, a functional F_0F_1-ATPase would be necessary for converting the energy stored in the electrochemical proton gradient into ATP synthesis. In the absence of a functional ATP synthase, the electrochemical proton gradient could not be abolished and this would inhibit the FHL reaction. This effect resembles the phenomenon of respiratory control observed in mitochondria [24]. Furthermore, in the ATPase mutants, the synthesis of the FHL complex might be downregulated. This is supported by previous studies [15], which have shown that the membrane fraction of the *E. coli* ATPase mutant AN936 (with a defect in the c-subunit of F_0), grown under fermenting conditions, did not have any hydrogenase activity.

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