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Dependence on the F_0F_1-ATP synthase for the activities of the hydrogen-oxidizing hydrogenases 1 and 2 during glucose and glycerol fermentation at high and low pH in *Escherichia coli*

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Abstract *Escherichia coli* has four [NiFe]-hydrogenases (Hyd); three of these, Hyd-1, Hyd-2 and Hyd-3 have been characterized well. In this study the requirement for the F_0F_1-ATP synthase for the activities of the hydrogen-oxidizing hydrogenases Hyd-1 and Hyd-2 was examined. During fermentative growth on glucose at pH 7.5 an *E. coli* F_0F_1-ATP synthase mutant (DK8) lacked hydrogenase activity. At pH 5.5 hydrogenase activity was only 20% that of the wild type. Using in-gel activity staining, it could be demonstrated that both Hyd-1 and Hyd-2 were essentially inactive at these pHs, indicating that the residual activity at pH 5.5 was due to the hydrogen-evolving Hyd-3 enzyme. During fermentative growth in the presence of glycerol, hydrogenase activity in the mutant was highest at pH 7.5 attaining a value of 0.76 U/mg, or ~50% of wild type activity, and Hyd-2 was only partially active at this pH, while Hyd-1 was inactive. Essentially no hydrogenase activity was measured at pH 5.5 during growth with glycerol. At this pH the mutant had a hydrogenase activity that was maximally only ~10% of wild type activity with either carbon substrate but a weak activity of both Hyd-1 and Hyd-2 could be detected. Taken together, these results demonstrate for the first time that the activity of the hydrogen-oxidizing hydrogenases in *E. coli* depends on an active F_0F_1-ATP synthase during growth at high and low pH.

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Introduction

Escherichia coli F_0F_1-ATP synthase is well known as the main membrane protein complex of bioenergetic relevance. The complex catalyzes ATP synthesis, the terminal step in oxidative phosphorylation. During fermentative growth, in the absence of aerobic or anaerobic respiration and oxidative phosphorylation, F_0F_1 functions as an ATP-driven proton pump to generate the proton motive force ($\Delta\mu_H^+$). During fermentation of sugars (glucose) this complex catalyzes ATP hydrolysis and H^+ movement associated with solute secondary transporters, especially the constitutive low affinity K^+ uptake TrkA system (Trchounian 2004; Kirakosyan et al. 2008). In addition, an interaction of the F_0F_1-ATP synthase with the low-affinity K^+ uptake system has been proposed for *Enterococcus hirae* (Trchounian and Kobayashi 1998). Little is known, however, regarding the role that the F_0F_1-ATP synthase might play in the function of anaerobic oxidation-reduction reactions such as hydrogen oxidation and evolution, for example by the formate hydrogenlyase pathway.

The formate hydrogenlyase complex is induced under fermentative growth when enterobacteria such as *E. coli* grow in the absence of exogenous electron acceptors (Rossmann et al. 1991). The complex is formed by the selenocysteine-and molybdenum-cofactor-containing formate dehydrogenase H and the [NiFe]-hydrogenase 3 (Hyd-3), and is responsible for disproportionation of formate to carbon dioxide and H_2 (Sawers et al. 1985).

This pathway is active at low pH and high formate concentration (Rossmann et al. 1991; Mnatsakanyan et al. 2004) and it is thought to provide a detoxification/deacidification system countering the build up of formate during fermentation. In addition to Hyd-3, which is encoded by the *hyc* operon (Sauter et al. 1992), there is [NiFe]-hydrogenase 4 (Hyd-4) encoded by the *hyf* operon which closely resembles Hyd-3. This system has been proposed by Andrews et al. (1997), along with the formate dehydrogenase H, to form a second energy-conserving formate hydrogenlyase complex. Hydrogen production appears to be ATP-dependent and it is increased when the $NADH/NAD^+$ ratio is high (Trchounian et al. 1997), for example during fermentation (De Graef et al. 1999).

Under certain conditions at neutral and slightly alkaline pH hydrogen evolution by *E. coli* is completely blocked by *N,N′*-dicyclohexylcarbodiimide (DCCD) (Bagramyan and Martirosov 1989; Bagramyan et al. 2002), which inhibits the F_0F_1-ATP synthase activity. Moreover, mutations in the *atp* operon encoding the catalytic subunits of the F_0F_1-ATP synthase lowered H_2 production (Trchounian et al. 1997; Mnatsakanyan et al. 2002). This confirms a role of F_0F_1 or $\Delta\mu_H^+$ in hydrogen metabolism. An independent study has also revealed a relationship between the F_0F_1-ATP synthase and the formate hydrogenlyase complex during thiosulfate reduction by *Salmonella typhimurium* (Sasahara et al. 1997). The activity of thiosulfate reductase and formate hydrogenlyase under anaerobic conditions was blocked by DCCD in the wild type and almost completely absent in *atp* mutants with non-functional F_0F_1-ATP synthase. A key role of the F_0F_1-ATP synthase in the H^+ movement accompanying certain anaerobic oxidation-reduction reactions has also been proposed and similar results have been observed for hydrogen production by *Rhodobacter sphaeroides* (Gabrielyan and Trchounian 2009) and recently for the archaeon *Thermococcus onnurineus*, which generates a $\Delta\mu_H^+$ driven by formate disproportionation via a formate hydrogenlyase complex (Kim et al. 2010).

As well as the hydrogen-evolving formate hydrogenlyase complex, *E. coli* possesses two other [NiFe]-hydrogenases, Hyd-1 and Hyd-2, which are hydrogen-oxidizing enzymes (Ballantine and Boxer 1985; Menon et al. 1991; King and Przybyla 1999; Richard et al. 1999). Synthesis of Hyd-1, encoded by the *hya* operon, is induced under anaerobic conditions at acidic pH (King and Przybyla 1999) and by the presence of formate but not nitrate (Laurinavichene et al. 2002). Although the precise physiological role of Hyd-1 is unclear, the enzyme is oxygen-stable and it catalyzes only hydrogen oxidation (Redwood et al. 2008; Lukey et al. 2010). Thus, it has likely an energy-conserving function. Hyd-2 is maximally synthesized and active in more alkaline medium (King and Przybyla 1999); this is in agreement with the reported pH optimum of the purified enzyme (Ballantine and Boxer 1985). Moreover, Hyd-2 activity was observed under more reducing conditions (Lukey et al. 2010) and is absent during aerobic growth (Redwood et al. 2008). Like Hyd-1, Hyd-2 has been proposed to have an energy-conserving function (Ballantine and Boxer 1985; Sawers et al. 1985).

Recently, it has been reported that glycerol can be metabolized by *E. coli* during fermentative growth with peptone (Dharmadi et al. 2006; Gonzalez et al. 2008; Hu and Wood 2010). As it has also been shown that mutations leading to defective Hyd-1 and Hyd-2 activity cause changes in the F_0F_1-ATP synthase activity dependent on pH (Blbulyan et al. 2011) it is therefore important to establish the effects of glycerol metabolism on hydrogen oxidation by Hyd-1 and Hyd-2 in *E. coli*.

In the present study the effects on Hyd-1 and Hyd-2 activity in a mutant lacking F_0F_1-ATP synthase after growth during glucose and glycerol fermentation were analyzed. The findings are consistent with a requirement for the F_0F_1-ATP synthase for hydrogen-oxidizing enzyme function, supporting a key role for both Hyd-1 and Hyd-2 in $\Delta\mu_H^+$ generation.

Materials and methods

Bacterial strains, their growth and preparation of cell extracts

The *E. coli* BW25113 (*lacI*^{q} *rrnB*$_{T14}$Δ*lacZ*$_{W116}$ *hsdR514* Δ*araBAD*$_{AH33}$ Δ*rha BAD*$_{LD78}$) wild type, JW0955 (Δ*hyaB*) (Trchounian and Trchounian 2009), MW1000 (Δ*hyaB* Δ*hybC*) (Trchounian et al. 2011) and DK8 (*bglR thil1 rel1* Δ(*uncB-uncC*) *ilv*::Tn*10*) (Klionsky et al. 1984) mutant strains were supplied by Prof. T.K. Wood (Texas A&M University, College Station, USA) and Dr. N. Mnatsakanyan (Texas Tech University, Lubbock, USA) and were used in this study.

Bacteria were grown under anaerobic conditions in peptone medium (20 g/l peptone, 15 g/l K_2HPO_4, 1.08 g/l KH_2PO_4, 10 g/l NaCl, at the indicated pH) supplemented with glycerol (10 g/l) or glucose (2 g/l) with incubation at 37 ^{0}C for 20–22 h (Bagramyan et al. 2002; Mnatsakanyan et al. 2002; Trchounian and Trchounian 2009).

For the preparation of crude extracts, all steps were carried out at 4 °C unless specifically stated otherwise. Harvested cells were washed in anaerobic MOPS-buffer (50 mM MOPS pH 7.5) and after centrifugation the cell pellet was suspended in 3 volumes of 50 mM MOPS pH 7.5 buffer including 5 μg DNase/ml and 0.2 mM phenylmethylsulfonyl fluoride. Typically 1–2 g wet weight of cells were disrupted by sonication (30 W power for 5 min with 0.5 s pulses). Unbroken cells and cell debris were removed

by centrifugation for 30 min at 50,000 x g and at 4 °C and the supernatant (crude extract) was either used immediately or stored at −80 °C until used.

Determination of total hydrogenase enzyme activity, non-denaturing polyacrylamide gel electrophoresis and in-gel hydrogenase activity staining

Hydrogenase enzyme activity (H_2-dependent reduction of benzyl viologen, BV) was measured according to (Ballantine and Boxer 1985) except that the buffer used was 50 mM MOPS, pH 7.0. The wavelength used was 578 nm and an E_M value of 8,600 M^{-1} cm^{-1} was assumed for reduced BV. One unit of activity corresponded to the reduction of 1 μmol of hydrogen per min. Non-denaturing polyacrylamide gel electrophoresis (PAGE) was performed using 7.5% (w/v) polyacrylamide gels, pH 8.5 and included 0.1% (w/v) Triton X-100 in the gels (Ballantine and Boxer 1985). Samples (50 μg of protein) were incubated with 5% (w/v) Triton X-100 prior to application to the gels. The detergent had no adverse effects on Hyd-1 and Hyd-2 enzyme activity. Hydrogenase activity-staining was done as described in (Ballantine and Boxer 1985) except that the buffer used was 50 mM MOPS pH 7.0. Each gel was incubated in an atmosphere of 95% nitrogen: 5% hydrogen for 8 h by which time the activity staining had run to completion.

Other methods

Protein concentration of crude extracts was determined by the method of Lowry et al. (1951) with bovine serum albumin as standard. Experiments were performed minimally three times and each time in triplicate. Enzyme activity is presented as standard deviation of the mean and is highly reproducible with generally not more than 5% deviation.

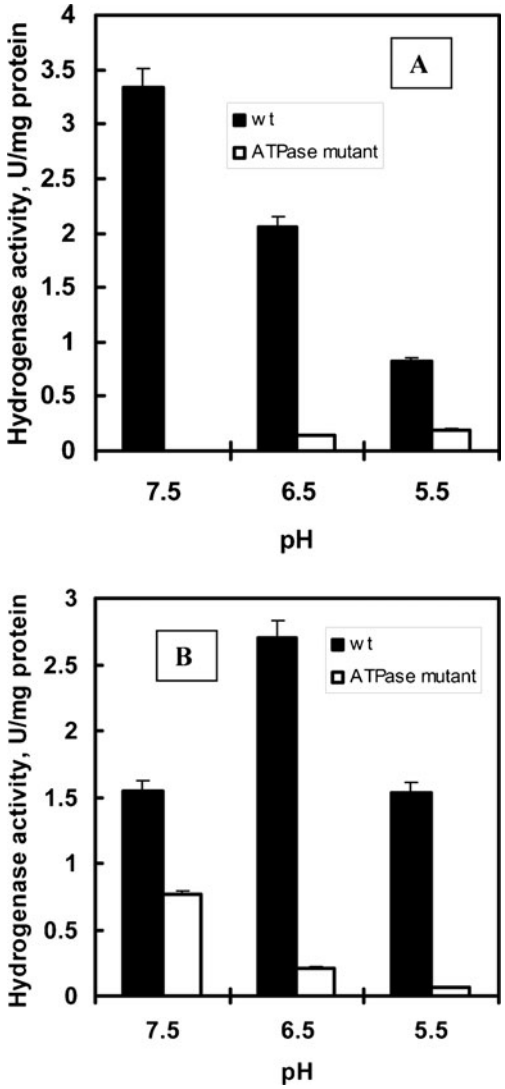

Fig. 1 Hydrogenase activity of *E. coli* BW25113 wild type and DK8 *atp* mutant strains grown and assayed at different pH on peptone medium supplemented with glucose (**a**) or glycerol (**b**)

Results and discussion

F_0F_1-ATP synthase is important for hydrogenase activity during glucose fermentation in *E. coli*

Initially, the hydrogenase enzyme activity in the mutant DK8, which is unable to synthesize the F_0F_1-ATP synthase, was determined during glucose fermentation and in response to the pH of the culture medium. Previous studies have shown that the *E. coli* F_0F_1-ATPase has the highest activity at alkaline pH (pH>7.0) (Bagramyan et al. 2002). Upon glucose fermentation at pH 7.5 no hydrogenase enzyme activity in DK8 could be measured while a high hydrogenase enzyme activity was measured in the wild type (Fig. 1a). The activities of Hyd-1 and Hyd-2 can be visualized after non-denaturing PAGE (Ballantine and Boxer 1985). Analysis of Hyd-1 and Hyd-2 enzyme activity by in-gel staining after non-denaturing PAGE revealed there were no activity bands corresponding to Hyd-1 or Hyd-2 in extracts derived from strain DK8 (Fig. 2a), which corroborated the findings of the enzyme assays. A hydrogenase-independent activity migrating near the top of the gel (marked by an asterisk in Fig. 2) and which was shown recently to be due to formate dehydrogenases N and O (Soboh et al. 2011), was unaffected by the *atp* mutation, and this activity acted as a loading control in further experiments.

Growth of DK8 in culture medium with pH 6.5 resulted in a hydrogenase activity of 0.15 U/mg while after growth with glucose at pH 5.5, a hydrogenase activity of 0.19 U/mg was determined. The wild type grown under the same conditions

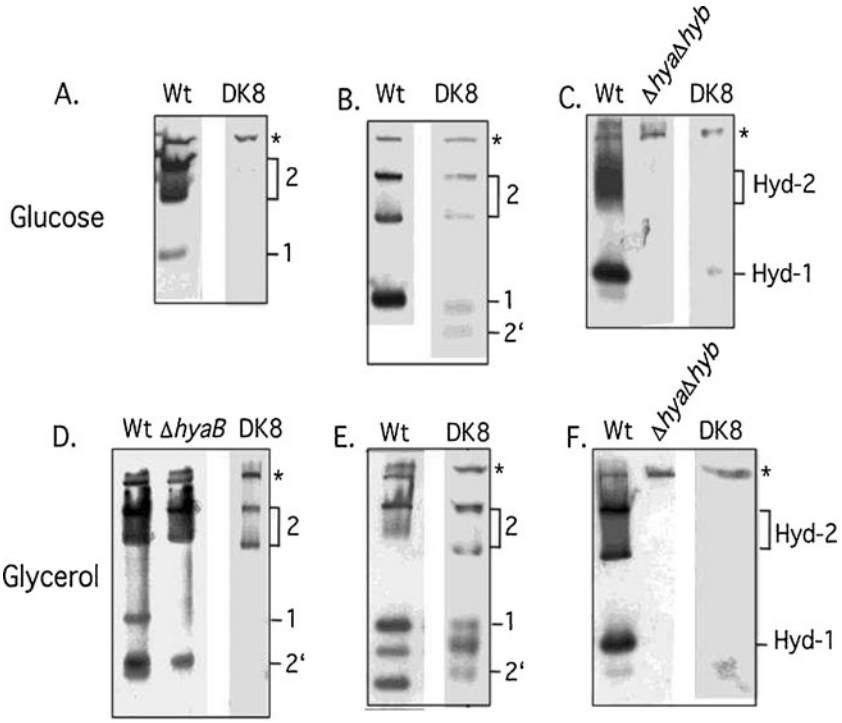

Fig. 2 Analysis of active Hyd-1 and Hyd-2 in *E. coli* by activity staining after non-denaturing-PAGE. Extracts derived from the strains indicated were separated by non-denaturing PAGE and subsequently stained for hydrogenase enzyme activity as described in the Materials and Methods. Strains were grown either with glucose (**a**, **b**, **c**) or glycerol (**d**, **e**, **f**) as indicated and at pH 7.5 (**a**, **d**), pH 6.5 (**b**, **e**) or pH 5.5 (**c**, **f**). Equivalent amounts of Triton X-100-treated crude extract (50 μg of protein) were applied to each lane. The activity bands corresponding to Hyd-1 and Hyd-2 are indicated as is the slowly migrating activity band (designated by an asterisk) that corresponds to a hydrogenase-independent activity of formate dehydrogenases N and O (Soboh et al. 2011) and which acted as a loading control. The band labeled 2′ represents a faster migrating degradation product of Hyd-2 (Ballantine and Boxer 1985) as demonstrated by the fact that it was also observed in a mutant unable to synthesize Hyd-1 (see middle lane in panel D)

had activities of 2.05 and 0.82 U/mg, respectively (Fig. 1a). These data indicate an inverse correlation between medium pH and hydrogenase activity in the DK8 mutant (Fig. 1a). In the in-gel hydrogenase activity assay, very weak activity bands corresponding to Hyd-1 and Hyd-2 were observed only after growth at pH 6.5 in DK8, while the wild type showed strong activity bands, particularly for Hyd-1 (Fig. 2b). After growth of DK8 at pH 5.5 activity of Hyd-2 could not be observed and that of Hyd-1 was barely detectable, indicating that the activity of both hydrogen-oxidizing enzymes was severely affected in the F_0F_1-ATPase mutant, particularly at extremes of pH (Fig. 2c). As a control, a mutant lacking the large subunits of both Hyd-1 and Hyd-2 (MW1000) failed to reveal any activity band (Fig. 2c). Notably, the in-gel staining data indicated that the weak hydrogenase-independent enzyme activity due to formate dehydrogenase, and designated by an asterisk, was observed under all growth conditions with equal intensity in the wild type and the F_0F_1-ATP synthase mutant. This indicates that the effects observed in DK8 grown on glucose at pH 5.5 did not affect all oxidoreductases and that the residual hydrogenase activity in the mutant was contributed by Hyd-3: due to the labile nature of its activity Hyd-3 cannot be observed in the gel-based assay (Sawers et al. 1985). These findings are also in accord with the strong inhibitory effect of DCCD on the activity of the third formate dehydrogenase H and H_2 production by *E. coli* at pH 7.5 (Trchounian et al. 1997; Bagramyan et al. 2002; Mnatsakanyan et al. 2004; Trchounian et al. 2011). This effect could be due either to the lack of F_0F_1-ATP synthase directly or may be mediated by a deficient $\Delta\mu_H^+$ (Trchounian 2004; Kirakosyan et al. 2008).

Hyd-activity is absent in an F_0F_1-ATP synthase deficient mutant during glycerol fermentation at low pH

During fermentation of peptone in the presence of glycerol it was observed that the H^+ efflux and F_0F_1-ATPase activity were lower than during glucose fermentation, but the F_0F_1-ATP synthase activity was highest at pH 5.5 (Blbulyan et al. 2011). However, a double mutant lacking both Hyd-1 and Hyd-2 had elevated F_0F_1-ATP synthase activity at pH 7.5 but not at pH 5.5 (Blbulyan et al. 2011). These results have been interpreted to indicate that the activity of the hydrogen-oxidizing Hyd-1 and Hyd-2 has a strong influence on the activity of F_0F_1-ATP synthase. During glycerol

fermentation at pH 7.5, the mutant DK8 had a lower hydrogenase-activity (by 50%) than the wild type (Fig. 1b). In-gel activity staining revealed that this activity was mainly due to Hyd-2 but not Hyd-1 (Fig. 2d). Under these conditions the activity of Hyd-3 is usually absent.

After growth of the wild type at pH 6.5 the total hydrogenase activity was similar to that during glucose fermentation and the mutant had a value of approximately only 10% of the activity in wild type extracts (Fig. 1b). The in-gel assay demonstrated that both Hyd-2 and Hyd-1 were active under these conditions (Fig. 2e). Growth of the wild type at pH 5.5 with glycerol resulted in a hydrogenase specific activity of~1.5 U/mg (Fig. 1b). In contrast, the hydrogenase activity of DK8 was barely detectable (Fig. 1b). In-gel activity-staining revealed that neither Hyd-1 nor Hyd-2 activity could be detected (Fig. 2f). Notably, however, the slowly migrating hydrogenase-independent activity of formate dehydrogenases N and O observed in these gels was still visible in the mutant, indicating that the effect of the *atp* mutation on Hyd-1 and Hyd-2 activities was specific. The data suggest that the requirement for the F_0F_1-ATP synthase activity for hydrogenase enzyme activity is the result of altered respiratory enzyme activity in *E. coli* in response to impaired oxidative phosphoryation (Noda et al. 2006).

Conclusions

The results with the *E. coli* F_0F_1-ATP synthase-negative mutant point to a requirement for active F_0F_1-ATP synthase for the activity of the hydrogen-oxidizing enzymes Hyd-1 and Hyd-2 during glucose and glycerol fermentation. Moreover, the data obtained indicate that there is an inverse correlation between hydrogenase activity and pH during fermentative growth on glucose and a direct correlation between hydrogenase activity and pH during glycerol fermentation. While both enzymes retain low activity during growth at pH 6.5 in the *atp* mutant, both are inactive at more extreme pH. These data demonstrate a metabolic link between the F_0F_1-ATP synthase activity and hydrogen-oxidizing activity and underscore the key role of Hyd-1 and Hyd-2 in energy conservation in fermenting *E. coli* cells.

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