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GLUCOSE CONCENTRATION DEPENDENT ATP-ASE ACTIVITY IN *ESCHERICHIA COLI* DURING FERMENTATION AND THE ROLE OF HIDROGENASE 4

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The dependence of *E. coli* BW25113 wild type's and JRG3621 (*hyfB-R)* mutant's membrane vesicles ATPase activity on glucose concentration and cooperation of F_OF_1-ATPase with Hyd-4 (*hyf*) under different conditions were studied. Different values of the *N,N'*-dicyclohexylcarbodiimide (DCCD)-sensitive ATPase activity were observed upon 0.2% and 0.8 % glucose fermentation conditions. These values depended on pH and K^+ content in the assay medium. DCCD-sensitive ATPase activity of 0.2 % glucose fermented *hyfB-R* mutant was lower 1.5-fold compared to wild type in K^+-free medium, and slightly increased by 100 mM K^+. The wild type demonstrated lower ATPase activity in both case, in K^+-free and in K^+-present media at pH 6.5, respectively ~1.5 and ~2-fold under 0.2% glucose fermentation, compared with pH 7.5. Unlike 0.2 % glucose fermentation, K^+ had suppressed effect on wild type's ATPase activity upon 0.8 % glucose fermentation at pH 6.5, whereas *hyfB-R* showed K^+ stimulated ATPase activity at the same conditions. The results pointed out the F_OF_1-ATPase's interaction with Hyd-4 and K^+ uptake systems upon 0.2% glucose fermentation at pH 7.5 which is important for understanding the role of $F_OF_1$and Hyd-4 under fermentation.

F_OF_1-ATPase – membrane proteins – hydrogenases – Hyd-4 – glucose fermentation – Escherichia coli

Ուսումնասիրվել է *E.coli*–ի BW25113 վայրի տիպի և *JRG3621 (hyfB-R)* մուտանտ շտամի թաղանթային բշտիկների ԱԵՖ-ազային ակտիվության կախվածությունը գլյուկոզի կոնցենտրացիայից և F_OF_1-ԱԵՖ-ազի ու Հիդ-4-ի փոխազդեցությունը խմորման տարբեր պայմաններում: 0.2 % և 0.8 % գլյուկոզի խմորման պայմաններում *N,N'*-դիցկլոհեքսիլկարբոդիիմիդ (ԴՑԿԴ)-զգայուն ԱԵՖ-ազային ակիվությունը՝ կախված ռեակցիոն միջավայրի *pH*-ից և K^+-ի պարունակությունից տարբեր է: 0.2 % գլյուկոզի խմորման պայմաններում (K^+-ից զուրկ միջավայրում) *E.coli*–ի hyfB-R մուտանտի ԴՑԿԴ-զգայուն ԱԵՖ-ազային ակտիվությունը՝ համեմատած վայրի տիպին՝ ցածր է 1.5 անգամ, և քիչ խթանված 100 մՄ K^+-ի ներկայությամբ: Վայրի տիպի ԱԵՖ-ազային ակտիվությունը pH 6.5-ի դեպքում՝ համեմատած pH 7.5-ի՝ ցածր է, ինչպես K^+-ից զուրկ, այնպես էլ K^+-պարունակող միջավայրերում, համապատասխանաբար ~1.5 և ~2 անգամ: 0.8 % գլյուկոզի խմորման դեպքում (pH 6.5) վայրի տիպի ԱԵՖ-ազային ակտիվությունը ընկճվել է K^+-ի ներկայությամբ, մինչդեռ նույն պայմաններում *hyfB-R* մուտանտում դիտվել է K^+-խթանված ԱԵՖ-ազային ակտիվություն: Այս տվյալները ցույց են տալիս 0.2 % գլյուկոզի խմորման պայմաններում pH 7.5 արժեքի դեպքում F_OF_1-ԱԵՖ-ազի և Հիդ-4-ի, ինչպես նաև K^+ կլանող համակարգերի փոխազդեցությունը, որն էլ կարևոր է խմորման պայմաններում F_OF_1-ի և Հիդ-4-ի դերը հասկանալու համար:

F_OF_1- ԱԵՖ-ազ – թաղանթային սպիտակուցներ – հիդրոգենազներ – Հիդ-4 – գլյուկոզի խմորում – Escherichia coli

Исследованы АТФ-азная активность мембранных везикул *E. coli* BW25113 дикого типа и мутанта JRG3621 (*hyfB-R)* в зависимости от концентрации глюкозы, и взаимодействие F_OF_1-АТФ-азы с Гид-4 при различных условиях среды. Мембранные везикулы *E. coli* проявляли разную *N,N'*-дициклогексилкарбодиимид (ДЦКД)-чувствительную АТФ-азную активность при сбраживании 0.2 % и 0.8 % глюкозы. АТФазная активность зависела от pH и наличия K^+ в реакционной среде. ДЦКД-чувствительная АТФ-азная активность *hyfB-R* мутанта при сбраживании 0.2 % глюкозы понижалась в 1.5 раза по сравнению с диким типом в отсутствие K^+ и возрастала в присутствие100 мМ K^+. Дикий тип демонстрировал более низкую АТФ-азную активность в ~1.5 раза при pH 6.5, по сравнению с pH 7.5 (~2 раз), как в присутствии K^+, так и при отсутствии данного иона. При сбраживании 0.8 % глюкозы (pH 6.5) K^+ подавлял АТФ-азную активность дикого типа, в отличие от 0.2 % глюкозы, а при тех же условиях *hyfB-R* показывал K^+-индуцированную АТФ-азную активность. Полученные результаты свидетельствуют о взаимодействиях F_OF_1-АТФ-азы с Гид-4 и с системами поглощения K^+ при сбраживании 0.2 % глюкозы при pH 7.5, что имеет важное значение для выявления роли F_OF_1 и Гид-4 при условиях брожения.

F_OF_1-АТФ-аза – мембранные белки – гидрогеназы – Гид-4 – брожение глюкозы – Escherichia coli

Escherichia coli has capacity to anaerobically ferment different carbon sources, such as glucose, glycerol, producing molecular hydrogen (H_2); the latter is considered an effective, ecologically clean and renewable energy source [13]. The genome of *E.coli* encodes four membrane-associated enzymes – [Ni-Fe]-hydrogenases (Hyd) [7, 15], two of which-Hyd-1(*hya*) and Hyd-2 (*hyb*) depend on carbon source and can function in different mode: during glucose or glycerol fermentation they operate in H_2 uptake or producing mode, respectively [15]. Hyd-3 (*hyc*) and Hyd-4 (*hyf*) are parts of two forms of membrane-associated formate hydrogen lyase complex (FHL); the complex containing Hyd-3 is considered as FHL-1, whereas Hyd-4 containing FHL is considered as FHL-2 [1, 6, 15]. Hyd-4 activity have been shown to depend on glucose concentration and seen during glucose low concentration [16, 18]. Interestingly, this finding is in favor with that glucose in higher concentrations can inhibit H_2 production [11].

Hyd-3 encoded by *hyc* operon which contains 9 genes is composed of various large and small subunits [8, 15]. Moreover, expression of this operon depends on pH_{out}, formate concetration, and molybdenium [10]. It was shown that Hyd-4 is primarily active at slightly alkaline pH and this enzyme is mainly responsible for H_2 production by *E. coli* [6]. The *hyf-focB* operon encodes 10 putative subunits of Hyd-4 and, moreover, the homologues of seven Hyd-3 subunits were encoded by *hyf* operon [1]. The *hyfR* gene encodes a format-sensitive regulatory protein, and terminal *focB* encodes putative formate transporter homologous with FocA [3, 15]. It is presumed that protein product of *hyfD*, *hyfE* and *hyfF* genes give H^+-translocating activity to Hyd-4 [1]. These Hyd enzymes require the proton F_OF_1-ATPase [4, 5]: some interaction of Hyd with F_OF_1 is suggested to maintain proton motive force [17]. In such respect, F_OF_1-ATPase activity dependence on glucose should be demonstrated.

Besides, under fermentative growth, in the absence of oxidative phosphorylation, F_OF_1 seems to be implicated as an essential part of H^+ movement and ATP hydrolysis, associated with secondary transporters, namely, the constitutive low affinity K^+ uptake TrkA system, responsible for K^+ accumulation in the cells [12, 14]. But the dependence of Hyd activity on K^+ is not clear.

The main goal of the present paper was to study dependence of F_OF_1-ATPase activity of *E. coli* on glucose concentration under different conditions and to determine Hyd-4 input in such dependence if any.

***Materials and methods*. Bacteria, bacterial growth, membrane vesicles**

The *E.coli* BW25113 wild type and JRG3621mutant strains were used in this study (tab. 1). Bacteria were grown under anaerobic conditions at 37°C for 18-20 h in high buffered peptone medium (20 g L^{-1} peptone, 15 g L^{-1} K_2HPO_4, 1.08 g L^{-1} KH_2PO_4, 10 g L^{-1}NaCl) with different concentrations of glucose (0.2% and 0.8%) in glass vessels with plastic press caps at different pHs (pH 7.5 and pH 6.5), as described elsewhere [4, 5]. The growth medium pH was determined by a pH-meter with a selective pH-electrode (HJ1131B, Hanna Instruments, Portugal) and adjusted using of 0.1 N HCl or 0.1 M NaOH if required.

Table 1. Characteristics of the *E. coli* wild type and mutant used

Strain	Genotype	Reference
BW 25113	$lacI^q$ $rrnB_{T14}$ Δ$lacZ_{W116}$ *hsdR514* Δ$araBAD_{AH33}$ Δ*rha* BAD_{LD78}	Sanchez-Torrez et al., 2003
JRG3621	MC4100 Δ(*hyfB-R*)::*spc*	Andrews et al., 1997

Membrane vesicles were isolated from lysozyme-treated bacteria by the method of Konings and Kaback [4].

ATPase assay

ATPase activity of membrane vesicles was determined from the amount of inorganic phosphate (P_i) produced in the reaction of membrane vesicles with 5 mM ATP (pH 7.5 and 6.5) [3, 5] in the assay mixture (50mM Tris-HCl buffer (pH 7.5 and 6.5) containing 1 mM $MgSO_4$). The ATPase activity was expressed in nM P_i/min (μg protein). P_i was determined spectrophotometrically (with UV-VIS Auto PS scanning spectrophotometer, LaboMed, USA) by the method of Tausski and Shorr as described [3, 5].

N,N'-dicyclohexcylcarbodiimide (DCCD) was used as an inhibitor of F_OF_1 [4, 12]. Membrane vesicles were treated by DCCD for 5 min prior to assay.

Other, Reagents and data processing

Protein levels were measured by the method of Lowry et al. (1951) using bovine serum albumin (BSA) as a standard [4]. All assays were done at 37 ^{0}C.

Agar, pepton, glucose, Tris-HCl (Carl Roth GmbH, Germany), ATP (Tris salt), DNAase 1, DCCD (Sigma, USA) and other reagents of analytical grade were used.

Average data obtained from 2-3 independent assays were represented and standard deviation of values did not exceed 3 %. For the differences between different series of experiments, Student validity criteria (p) were determined using Microsoft Excel 2010; the difference was valid if $p<0.05$.[4, 5]

***Results and Discussion.* ATPase activity of glucose (0.2% and 0.8%) fermented *E. coli* membrane vesicles at pH 7.5.**

The overall ATPase activity of membrane vesicles from glucose-fermented *E. coli* BW25113 wild type and JRG3621 (*hyfB-R)* mutant and its inhibition by DCCD were investigated at different pHs. It is worth to mention that at pH 7.5 upon 0.2% glucose fermentation ATPase activity of wild type of 350 nM P_i/min (μg protein) was similar with previous results [3]. DCCD-sensitive ATPase activity was increased ~1.4-fold by K^+ (100 mM) compared to that at the absence of K^+ (fig.1a). This finding pointed out a close relationship of *E.coli* F_OF_1 and TrkA under certain conditions. Note, 0.2 mM DCCD strongly inhibited (5-fold, $p< 0.001$) ATPase activity in K^+-free assay buffer, but inhibition by DCCD was higher (~7-fold, $p<0.002$) at the presence of K^+ (tab. 2). Moreover, DCCD-sensitive ATPase activity of 0.2 % glucose fermented *hyfB-R* mutant was 5-fold in comparison with wild type in K^+-free medium at pH 7.5. It was also shown that ATPase activity of *hyfB-R* membrane vesicles increased ~1.2-fold ($p>0.05$) by 100 mM K^+ (fig.1a). These data confirmed a role of Hyd-4 during glucose fermentation at slightly alkaline pH that is in maintaining of proton-motive force as suggested before [12, 17, 18]. It might be explained by mediated role of this Hyd enzyme in the formation of F_OF_1-TrkA complex and in direct transfer of energy from F_OF_1 to TrkA [12].

In contrast to 0.2% glucose fermentation at pH 7.5, during growth on 0.8% glucose in K^+ -free medium, the overall and DCCD-sensitive ATPase activity of wild type, was approximately 18-22% higher (fig.1a,1b). Note, DCCD inhibited significantly (~7-fold, $p<0.001$) and (~4-fold, $p<0.002$) ATPase activity of wild type in K^+ -free and K^+-containing medium, respectively (tab. 2). However, it was worth to mentioning that membrane vesicles of 0.8% glucose fermented wild type demonstrated lower DCCD-sensitive ATPase activity in K^+-containing medium compared with K^+ -free medium (fig.1b). These results indicated that F_OF_1-ATPase activity is glucose-concentration dependent: F_OF_1 is active mainly at low concentration of glucose (0.2%) at pH 7.5. Probably, at higher concentration of glucose (0.8%) there is no link between F_OF_1 and TrkA. Then, upon 0.8 % glucose at pH 7.5 the membrane vesicles of *hyfB-R* demonstrated ~2.2-fold ($p<0.01$) lower ATPase activity in K^+ -free medium, compared to wild type, and100 mM K^+ had no effect on ATPase activity (fig. 1b). These results are likely to those suggesting a different mode of TrkA depending on pH [14] and a role of Hyd-4 in association of F_OF_1 with TrkA complex [12, 17, 18].

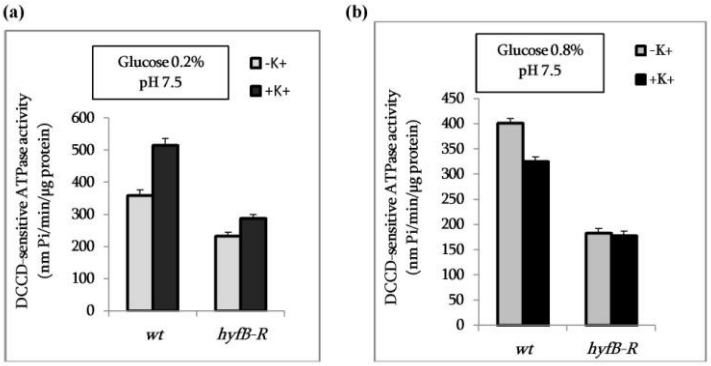

Fig. 1. ATPase activity of membrane vesicles of *E. coli* wild type (wt) and *hyfB-R* mutant grown on peptone medium supplemented with 0.2% glucose (a) and 0.8% glucose (b) at pH 7.5. The DCCD (0.2mM) was added into the assay medium when indicated. The assays pH was the same as growth pH. For the strains see tab. 1; for others, see Materials and methods.

ATPase activity of glucose (0.2% and 0.8%) fermented *E. coli* membrane vesicles at pH 6.5.

The ATPase activity of *E.coli* wild type and *hyfB-R* was determined upon different concentration of glucose (0.2% and 0.8%) at pH 6.5. Under 0.2% glucose fermentation membrane vesicles of wild type demonstrated lower ATPase activity in both cases, in K^+-free and in K^+-contented media at pH 6.5, compared with those at pH 7.5 (fig.2a, comp. fig. 1a). 100mM K^+ was slight effect (~1.2-fold, $p<0.02$) on wild type DCCD-sensitive ATPase activity, whereas DCCD markedly inhibited ATPase activity ~7-fold ($p<0.001$) and ~12-fold ($p<0.001$) in K^+-free and inK^+-contented assay buffers, respectively (tab. 2). Mutant, also showed similar DCCD-sensitive ATPase activity, as wild type, in K^+-free medium (fig. 2a). Note, that K^+ had no effect on ATPase activity of *hyfB-R*; moreover, it was decreased in K^+-containing medium (fig.2a). The obtained results allow suggestion of a mediated role of Hyd-4 for F_OF_1-TrkA association.

Then, in 0.8% glucose fermented cells, compared with 0.2% glucose fermented cells, DCCD-sensitive ATPase activity at pH 6.5 in K^+-free medium, was lower ~1.2-fold ($p>0.05$) in wild type and ~4-fold ($p<0.01$) in *hyfB-R* mutant (fig. 2a, 2b). It should be noted that in K^+-containing medium membrane vesicles of wild type showed ~1.6-fold ($p<0.02$) lower ATPase activity compared with that in K^+-free medium, whereas in mutant

K^+ increased ATPase activity ~1.7-fold ($p<0.05$) (fig. 2b). These data pointed out a relationship between F_OF_1-ATPase and K^+-uptake upon 0.8% glucose fermentation at pH 6.5 but Hyd-4 had no any role. Note, the DCCD-sensitive ATPase activity of mutant was ~30 % of wild type activity (fig. 2a). DCCD suppressed ATPase activity of wild type ~7-fold ($p<0.02$) and ~2.4-fold ($p<0.01$), respectively, in K^+-free and K^+-containing media

In comparison with wild type, the inhibition of mutant's ATPase activity by DCCD in K^+-present medium was significant - ~8-fold ($p<0.02$) (see tab. 2).

Table 2. DCCD-inhibited ATPase activities of membrane vesicles of *E. coli* wild type and mutant grown under different concentrations of glucose fermentation (tab. 1).

Strain	DCCD-inhibited ATPase activity, %*							
	pH 7.5				pH 6.5			
	0.2% Glucose		0.8% Glucose		0.2%Glucose		0.8%Glucose	
	$-K^+$	$+K^+$	$-K^+$	$+K^+$	$-K^+$	$+K^+$	$-K^+$	$+K^+$
wt	79 ± 1	85 ± 1	86 ± 1	73 ± 2	84 ± 1	92 ± 1	84 ± 1	63 ± 2
hyfB-R	82 ± 1	87 ± 1	92 ± 1	82 ± 2	85 ± 2	84 ± 1	67 ± 1	87 ± 2

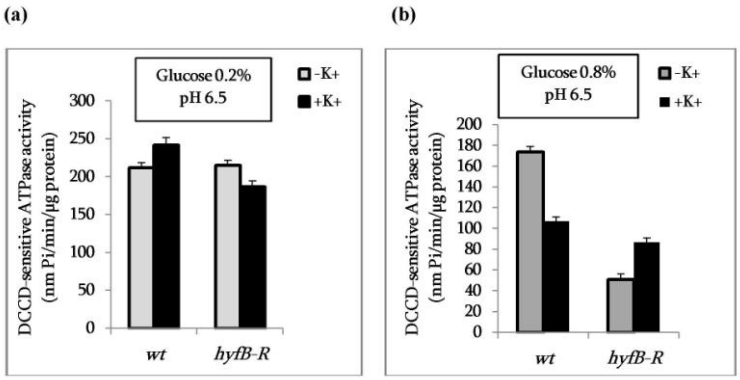

Fig. 2.ATPase activity of membrane vesicles of *E. coli* wild type and *hyfB-R* mutant grown on peptone medium supplemented with 0.2% glucose (a) and 0.8% glucose (b) at pH 6.5.
For the others, see ***Materials and methods*** and legends to fig. 1.

The present results allow suggesting that high concentration of glucose (0.8%) impacts on *E. coli* F_OF_1-ATPase activity and its association with Hyd-4, as well as with secondary transporters, such as TrkA. 100mM K^+ increased ATPase activity of wild type only at glucose limited conditions under fermentation at pH 7.5 and pH 6.5.

These findings support the F_OF_1–TrkA association formed during fermentative conditions at low concentration of glucose (0.2 %) and at alkaline pH in *E. coli* [12, 14, 17]. It was suggested that Hyd-4 could be linked to the F_OF_1-ATPase supplying reducing equivalents for energy transfer [12, 17, 18]. The lower ATPase activity of *hyfB-R* upon high concentration of glucose (0.8 %) at pH 6.5 can be presumably explained by high production of formate, which is acidificated intracellular medium and as a result F_OF_1-ATPase switches on regulatory mechanism suppressing ATPase activity.

Moreover, the lower ATPase activity of wild type in presence of K^+ under 0.8 % glucose fermentation at both pH 7.5 and pH 6.5, also showed that functional link between F_OF_1-ATPase and secondary transport systems like TrkA is depended on glucose concentration and external pH.

Thus, the reported results pointed out the interaction of the F_OF_1-ATPase with Hyd-4 and K^+ uptake systems which is important for understanding the role of F_OF_1 and Hyd-4 under fermentation.

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