Chromogenic assessment of the three molybdo-selenoprotein formate dehydrogenases in Escherichia coli

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Abstract

Escherichia coli synthesizes three selenocysteine-dependent formate dehydrogenases (Fdh) that also have a molybdenum cofactor. Fdh-H couples formate oxidation with proton reduction in the formate hydrogenlyase (FHL) complex. The activity of Fdh-H in solution can be measured with artificial redox dyes but, unlike Fdh-O and Fdh-N, it has never been observed by chromogenic activity staining after non-denaturing polyacrylamide gel electrophoresis (PAGE). Here, we demonstrate that Fdh-H activity is present in extracts of cells from stationary phase cultures and forms a single, fast-migrating species. The activity is oxygen labile during electrophoresis explaining why it has not been previously observed as a discreet activity band. The appearance of Fdh-H activity was dependent on an active selenocysteine incorporation system, but was independent of the [NiFe]-hydrogenases (Hyd), 1, 2 or 3. We also identified new active complexes of Fdh-N and Fdh-O during fermentative growth. The findings of this study indicate that Fdh-H does not form a strong complex with other Fdh or Hyd enzymes, which is in line with it being able to deliver electrons to more than one redox-active enzyme complex.

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1. Introduction

Formate and hydrogen are important electron donors, as well as key fermentation products, in the metabolism of numerous anaerobic bacteria and archaea [1]. During anaerobic growth, the model bacterium Escherichia coli synthesizes three formate dehydrogenases (Fdh) and up to four [NiFe]-hydrogenases (Hyd) [2]. The Fdh enzymes include Fdh-H (H for hydrogen, i.e. fermentation), Fdh-N (N for nitrate respiration) and Fdh-O (O for aerobic growth) to signify the conditions under which they were originally identified and that were primarily used for their characterization [2]. All three Fdh have a large subunit that contains both a selenocysteine residue and a molybdenum cofactor in their active site. Both of these cofactors are directly involved in catalysis [2]. This catalytic subunit also includes a [4Fe-4S] cluster that mediates electron transfer between the active site and [4Fe-4S] clusters of an electron-transferring small subunit. In contrast, the four Hyd enzymes include Hyd-1 and Hyd-2, which are respiratory enzymes that primarily couple hydrogen oxidation to quinone reduction, while Hyd-3, together with Fdh-H, forms part of the formate hydrogenlyase (FHL) complex, which catalyzes H₂ production from formate [2]. Hyd-4 is also proposed to form a complex including Fdh-H, which is related to FHL [3]. All seven enzymes form key components of membrane-associated multi-protein complexes and these are depicted schematically in Fig. 1.

Biocatalytic, and particularly physiological, analysis of individual Fdh enzymes has been hampered by the fact that activity can only efficaciously be determined using artificial redox dyes. Nevertheless, use of dyes such as benzyl viologen (BV) and phenazine methosulfate/2,6-dichlorophenolindophenol (PMS/DCPIP) for enzyme assays in solution, along with selenopolypeptide analyses, demonstrated that Fdh-H and Fdh-N are indeed distinct enzymes [4,5]. Subsequent biochemical [6,7] and molecular biological studies [8] showed that Fdh-O (encoded by fdoGHI), although highly similar to Fdh-N (encoded by fdnGHI), is distinct from it and revealed that Fdh-O probably represents the aerobic formate-oxidizing activity originally identified in the early 1950s [9].

Development of an effective and facile in-gel activity-staining procedure has proved useful in the study of the physiology of the Hyd enzymes, particularly with regard to what governs their synthesis and activity [10]. Although the activities of both Fdh-O and Fdh-N have been determined with PMS/nitroblue tetrazolium (NBT) [10], to date, however, no evidence has ever been provided that the activity of the highly labile Fdh-H enzyme [4], encoded by the fdhF gene [2], withstands polyacrylamide gel electrophoretic (PAGE) separation from other anaerobic enzyme complexes. Moreover, no systematic in-gel activity analysis of the Fdh enzymes of E. coli during fermentative growth has been conducted. In this study we identify conditions that reveal Fdh-H enzyme activity.
after non-denaturing PAGE. The Fdh-H activity is very oxygen-labile, perhaps explaining why previous studies have failed to identify it after gel electrophoresis. Furthermore, these studies identify previously unobserved active enzyme complexes of Fdh-O and Fdh-N during fermentative growth of *E. coli*.

### 2. Materials and methods

#### 2.1. Strains and growth conditions

The strains used in this study are listed in Table 1. *E. coli* strains were routinely grown at 37 °C on LB-agar plates or with shaking in LB-broth [11]. Anaerobic growths for the preparation of extracts to study enzyme activity were performed at 37 °C as standing liquid cultures [12] in M9-minimal medium containing 1/2 M9 salts[11], 2 mM MgSO₄, 0.1 mM CaCl₂, 3 μM thiamine hydrochloride, trace element solution SL-A [13], and 0.8% (w/v) glucose. Kanamycin, when required, was used at the final concentration of 50 μg/ml.

Cells were harvested anaerobically by centrifugation at 15,000 g for 15 min and at 4 °C after the cultures had reached an OD₆₀₀ nm of either 0.6–0.8 (exponential phase) or 1.2 (stationary phase). Cell pellets were used immediately or stored at −20 °C until use.

#### 2.2. Strain construction

Strains were constructed using P1kc-mediated phage transduction [11] to introduce the respective defined deletion mutation from the appropriate strains obtained from the Keio collection [14]. When required, the plasmid pCP20 was used to remove the kanamycin antibiotic resistance cassette as described[15].

#### 2.3. Preparation of crude cell extracts

Wet cell paste was re-suspended at a ratio of 1 g/3 ml in 50 mM MOPS pH 7 including 5 μg DNase ml⁻¹ and 0.2 mM phenylmethyl-sulfonyl fluoride. Cells were disrupted aerobically by sonication (30 W power for 5 min with 0.5 s pulses) and then placed immediately under a N₂ atmosphere. Unbroken cells and debris were removed by centrifugation for 30 min at 50,000g at 4 °C. The resultant supernatant was termed the crude extract and was used for all studies reported herein.

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**Table 1**

| Strain     | Genotype                                      | Reference |
|------------|-----------------------------------------------|-----------|
| MC4100     | F-, andD139, Δ[argF-lac]J169, λ-, rpsL150, relA1 deoC1, fhlDS301, Δ(fruK-yedR)725(fruA25), rbsR22, Δ(fimB-fimF)IS32(3; IS1) | [28]      |
| FTD147     | MC4000 ΔhyaB ΔhyaC ΔhyaE                      | [29]      |
| FM460      | MC4100 ΔselC (Kan⁶)                           | [30]      |
| CP585      | MC4000 ΔfdhF                                  | This study|
| CP773      | MC4000 ΔhydA ΔhydC                            |           |
| CP1002     | CP734 ΔhybB (Kan⁶)                            |           |
| CP1010     | CP734 ΔfdhF (Kan⁶)                            | This study|
| HD705      | MC4000 ΔhyaE                                  | [32]      |
| JW3852     | BW25113 ΔfdhE (Kan⁶)                         | [14]      |
| SH1        | FTD147 ΔselC (Kan⁶)                          |           |
| SH173      | FTD147 ΔfdgC                                 | This study|
| SH174      | FTD147 ΔfdgC                                 |           |
| SH175      | SH173 ΔfdgC (Kan⁶)                           |           |
| SH1796     | SH175 with Kan⁶-cassette removed              |           |
| SH200      | SH196 ΔselC (Kan⁶)                           |           |
2.4. Polyacrylamide gel electrophoresis, activity staining, and protein determination

Unless stated otherwise, non-denaturing polyacrylamide gel electrophoresis (PAGE) was carried out under anaerobic conditions in a Coy™ chamber in an atmosphere of 95% N2:5% H2. Non-denaturing PAGE was performed using 6% (w/v) polyacrylamide and gels were maintained under anaerobic conditions prior to staining. Hydrogenase activity-staining was done in 50 mM MOPS buffer pH 7.0, as described [16], and included 0.5 mM benzylioligo (BV) and 1 mM 2,3,5-triphenyltetrazolium chloride (TTC). Gels were incubated at RT under an atmosphere of 100% highly pure hydrogen gas [10]. Alternatively, staining was done in a 100% hydrogen atmosphere using 0.3 mM phenazine methosulfate (PMS) as mediator and 0.2 mM nitroblue tetrazolium (NBT) as redox dyes [10]. Unless otherwise stated, formate (50 mM concentration) was added to the activity staining buffer to visualize formate dehydrogenase activity [17,18]. Determination of protein concentration was done as described [19].

3. Results and discussion

3.1. Active formate dehydrogenase H is present in stationary phase cells

Fdh-H enzyme activity is oxygen-labile [4] and has so far never been observed after non-denaturing PAGE. A previously unobserved, fast-migrating and formate-dependent enzyme activity was revealed after separation of enzyme complexes in extracts derived from the parental strain MC4100 by non-denaturing PAGE (Fig. 1A, lane 1). The gel was run under anaerobic conditions and the enzyme activity was stained with PMS/NBT using a combination of 50 mM formate and a H2 atmosphere for the activity staining procedure. The enzyme activity appeared within seconds of placing the gel in the formate-containing activity stain, without the necessity for bubbling with a 100% H2 atmosphere. This enzyme activity was observed in extracts derived from stationary phase cells only and not in extracts derived from cells harvested in the exponential phase (compare lane 1 in Fig. 2A and B). Moreover, no activity band could be observed when formate was omitted from the staining procedure (Fig. 2C). Both of these findings are consistent with this activity being due to the fdhF gene product, Fdh-H; expression of fdhF occurs preferentially in the stationary phase of growth [2]. To prove that this enzyme activity was indeed due to Fdh-H, an extract derived from an E. coli selC mutant, which cannot insert selenocysteine into any of the Fdh enzymes [2], was shown to be devoid of the activity band (Fig. 2A, lane 3). Notably, the activity bands corresponding to Fdh-N and Fdh-O were also absent from strain FM460 (ΔselC). Introduction of a ΔfdhF allele into the parental strain MC4100, delivering strain CP585 (ΔfdhF), as well as into strain CP734 (ΔhyAB, ΔhyBC), delivering strain CP1010 (ΔhyAB, ΔhyBC, ΔfdhF), also prevented synthesis of the enzyme activity (Fig. 2A, lanes 11 and 12), again consistent with this species being due to Fdh-H.

Next, we examined whether Fdh-H activity was reliant on the presence of either Fdh-O or Fdh-N. Deletion of the fdgO gene or the fdgN gene, encoding the catalytic subunit of Fdh-O or Fdh-N, respectively, or indeed both genes together, did not affect the appearance of the Fdh-H activity (Fig. 2A, compare lanes, 4, 5 and 6). Introduction of the ΔselC allele into the Fdh-N-ΔO- Fdh-O double null strain, delivering strain SH200 (Table 1), abolished Fdh-H activity, as anticipated (Fig. 2A, lane 7). Finally, analysis of a mutant lacking the fdhE gene, which encodes an iron-sulfur protein with an essential function in the maturation of the periplasmically-oriented Fdh-O and Fdh-N, but not that of Fdh-H [20,21], exhibited a clear activity band due to Fdh-H, but completely lacked enzyme activity for either Fdh-N or Fdh-O (Fig. 2A, lane 13). Together, these data identify the fast-migrating formate-dependent enzyme activity as Fdh-H and demonstrate that activity of the enzyme is high in the stationary phase of growth and is independent of the other two Fdh enzymes. The latter observation is consistent with earlier findings [2,4].

3.2. Barriers to determining Fdh-H in-gel activity

The oxygen-labile nature of the Fdh-H enzyme activity during purification has been well documented [4,22,23]. To determine whether the Fdh-H activity detected after anaerobic non-denaturing PAGE could also be visualized after performing the electrophoresis under aerobic conditions, the same samples as those shown in Fig. 2A were subjected to aerobic non-denaturing PAGE (Fig. 2D). No Fdh-H enzyme activity could be observed. Despite the cells being briefly exposed to O2 during disruption, Fdh-H activity was retained in the crude extract. It appears that separation of the enzyme from the rest of the FHL complex during electrophoresis renders it more sensitive to the deleterious effects of oxygen [4,22] (R.G. Sawers unpublished data).

When the same crude extracts from stationary phase cells were separated by anaerobic non-denaturing PAGE and the gel was incubated in a H2 atmosphere with 50 mM formate as electron donor and BV/TTC as electron acceptors, no Fdh-H enzyme activity band could be observed, despite the detection of the well-characterized bands of the three hydrogenases Hyd-1, Hyd-2 and Hyd-3 and weak activities due to Fdh-N and Fdh-O (Fig. 3A). The redox potential of H2-saturated, formate buffer used for the BV/TTC activity stain (E = −320 mV) was significantly lower than that of the PMS/NBT stain (E = −230 mV), suggesting that the Fdh-H enzyme catalyzes electron transfer more efficiently to the buffer with the more positive redox potential.

3.3. Fdh-H is active in the absence of a functional Hyd-3

The Fdh-H enzyme has, so far, only been observed to be associated with the FHL complex [24], although it has been suggested that it might also form a complex with the hyf gene products, encoding Hyd-4 [3]. The fdhH gene, which encodes Fdh-H, is located separately, and in isolation, from the hyf genes and from those encoding Hyd-3 and the FHL complex, which would be in accord with this proposal. This is also in line with our finding here that Fdh-H activity, after separation in non-denaturing PAGE, does not co-migrate with Hyd-3 activity (Fig. 2A). A recent study has demonstrated that if the fdhH gene is deleted, the in-gel activity of Hyd-3 is still measurable but is significantly reduced in intensity [10]. This result was confirmed in the current study (Fig. 3, compare lanes 1, 8, 11 and 12). To test whether the in-gel Fdh-H activity is influenced by the absence of Hyd-3, an extract from HD705 (ΔhyEC) was prepared and after non-denaturing PAGE the gel was stained for Fdh-H activity (Fig. 2A, lane 10). The result clearly demonstrates that Fdh-H activity is independent of the presence of Hyd-3 activity in stationary phase E. coli cells. Moreover, Fdh-H activity could also be visualized in a mutant lacking the HyEC protein (Fig. 2A, lane 9), which has been proposed to act as the small subunit channeling electrons from Fdh-H to the FHL complex [24]. It should be noted that the fdhH gene product has an iron-sulfur cluster and when purified anaerobically the protein is able to transfer electrons to redox dyes [4]. Together, these results indicate that Fdh-H retains activity in the absence of the catalytic subunit HyEC of the Hyd-3 enzyme or in strains that lack HyEC, the protein that is presumed to act as the docking site for Fdh-H on the FHL complex [24].
3.4. Active Fdh-O and Fdh-N sub-complexes in exponential phase cells

While the activities of both Fdh-N and Fdh-O are detectable in extracts of exponential and stationary phase cells, activity of both enzymes is highest in exponential phase cells (Fig. 2B). This contrasts with the activity of Fdh-H, which is more active in stationary phase cells (see Fig. 2A). It was noted that two new, faster migrating sub-complexes that were dependent on the presence of the fdoG gene, encoding the catalytic subunit of H2-dependent enzyme complex. The migration position of two faster-migrating Fdh-O-dependent enzyme complexes observed in exponentially growing cells (B) is indicated within the figure.

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These two activity bands were observed in extracts of a strain lacking Fdh-N (Fig. 2B, lane 4), but not in extracts of strains lacking Fdh-O (Fig. 2B, lane 5), strongly suggesting that they are additional complexes of Fdh-O. Consistent with this suggestion, these new activity bands were absent in extracts of the selC mutant FM460 (Fig. 2B, lane 3). It is currently unclear whether these active species are sub-complexes of a presumptive trimer-of-trimers (3 x FdnGHI), as has been observed for Fdh-N [25], or whether they are complexes of Fdh-O interacting with other redox enzymes.

A rather diffuse additional, faster-migrating activity band that was dependent on Fdh-N was also observed when extracts from stationary phase cells were subjected to the activity staining procedure using BV/TTC as electron acceptor (Fig. 3A, lanes, 2, 5, 8, 9 and 11). This additional activity band was almost completely absent in extracts derived from strain SH173 (Fig. 3A, lane 4), which, in addition to lacking genes encoding the catalytic subunits of Hyd-1 through -3, is also devoid of fdnG (Table 1). Notably, the activity was dependent upon formate (Fig. 3B). Thus, despite the Fdh-O and Fdh-N enzymes sharing high levels of amino acid sequence similarity, e.g. the catalytic subunits of both enzymes share 76% identity, the fact that the newly identified Fdh-O and Fdh-N complexes specifically reduced PMS/NBT and BV/TTC, respectively, indicates that they differ in the substrates to which they deliver electrons. In the case of Fdh-O this might be related to the fact that the enzyme is synthesized under aerobic as well as anaerobic conditions [7], and that it forms ‘super-complexes’ with cytochrome o and cytochrome d oxidases [26]. Consequently, Fdh-O possibly uses ubiquinone rather than menaquinone as physiologically required electron donor, as has been previously suggested [27], and hence its preferential use of the redox dye combination of PMS/NBT, presumably because

hence its preferential use of the redox dye combination of PMS/NBT and BV/TTC. The latter combination functions optimally for the determination of hydrogenase activity. Fdh-H activity was shown to depend on formate and the physiological role for aerobic formate dehydrogenase, J. Bacteriol. 177 (1995) 18213–18218.

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