FOR THE RECORDS

Title:

Atypical effect of temperature tuning on the insertion of the catalytic iron-sulfur centre in a recombinant [FeFe]-hydrogenase

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Running title:

Temperature effect on [FeFe]-hydrogenase expression

Total number of manuscript pages: 17

Figures: 1

Tables: 1

Abstract

The expression of recombinant [FeFe]-hydrogenases is an important step for the production of large amount of these enzymes for their exploitation in biotechnology and for the characterisation of the protein-metal cofactor interactions. The correct assembly of the organometallic catalytic site, named H-cluster, requires a dedicated set of maturases that must be co-expressed in the microbial hosts or used for *in vitro* assembly of the active enzymes. In this work, the effect of the post-induction temperature on the recombinant expression of CaHydA [FeFe]-hydrogenase in *E. coli* is investigated. The results show a peculiar behaviour: the enzyme expression is maximum at lower temperatures (20°C), while the specific activity of the purified CaHydA is higher at higher temperature (30°C), as a consequence of improved protein folding and active site incorporation.

Keywords

[FeFe]-hydrogenases; Recombinant expression; Bio-hydrogen; metalloenzyme.

50-75 words statement

Post-induction temperature severely influences the recombinant expression in *E. coli* of the [FeFe]-hydrogenase CaHydA, a metalloenzyme hosting the peculiar catalytic centre H-cluster. The best protein yield is observed at lower temperature (20°C), while the best specific activity is obtained at higher temperature (30°C), which is atypical in comparison to the usual trend for recombinant holo-enzymes.

Introduction

[FeFe]-hydrogenases are the enzymes that reversibly catalyse the production of molecular hydrogen, following the reaction $2H^+ + 2e^- \rightleftarrows H_2$ [1]. They are widely distributed among prokaryotes and eukaryotes and are essential in the energy metabolism of such organisms, being usually involved in the dissipation of excess of reducing equivalents in the cell. A significant biotechnological interest has been directed to their exploitation in new, clean and efficient industrial processes for the production of H_2, to be used as a valuable fuel and industrial intermediate [2-7].

The production of [FeFe]-hydrogenases by recombinant techniques has become relevant for several reasons. First of all, the recombinant techniques allow the manipulation of the protein: 1) by inserting tag sequences that facilitate purification [8-10], which is highly desirable given the need to work under anaerobic conditions; 2) by inserting single mutations for the study of target residues [11-14]; 3) by generating random mutations for the study of complex features [15-19]. Moreover, recombinant expression usually grants the availability of large amount of enzyme that are required for the characterisation [9,20-24] and for the development of possible future applications [5,6,25]. Recombinant expression has also paved the way to study the mechanisms of the insertion of the catalytic centre H-cluster in the enzyme [FeFe]-hydrogenases, the so-called maturation [8,21,26-29].

The recombinant systems that have been developed so far are either cell-hosted or cell-free. The systems that are cell-hosted are carried out in three different hosts: *Escherichia coli* [8,10,20], *Clostridium acetobutylicum* [9,30] and *Shewanella oneidensis* [31]. The cell-free systems are based on the *in vitro* insertion of the H-cluster into an apo-[FeFe]-hydrogenase: in some cases the maturases are added [32-35], while in others the H-cluster is inserted as a chemically synthesised complex [36,37].

Given the simplicity and the technological availability of all the components, the expression system for *E. coli* has been widely developed and used. In previous reports, the effect of several parameters has been optimised, but the temperature was never analysed in details, as most authors carried out the experiments at room temperature [8,10,20].

In this work, we report on the effect of the post-induction temperature on the recombinant expression of *Clostridium acetobutylicum* CaHydA [FeFe]-hydrogenase in *E. coli* with a C-terminal Strep-tagII.

Results and Discussion

The effect of the post-induction temperature was assayed by SDS-PAGE (Fig. 1A) that allows to observe the levels of expression of the maturases CaHydF and CaHydG, as well as western blot stain against Strep-TagII (Fig. 1B) that specifically discriminates the level of CaHydA. From the functional point of view, the total H_2 evolution activity was assayed on whole cells by gas chromatography (Fig. 1C).

These results (Fig. 1A, 1B and 1C) clearly show that the amount of the maturases, the amount of CaHydA and the total hydrogenase activity in whole cells reach a maximum at 20°C, suggesting this temperature as the best condition.

To confirm the results observed in whole cells, the enzyme was anaerobically purified by Strep-tagII affinity chromatography and the yield of pure protein and specific hydrogenase activity were measured as previously described [18].

The characterisation of the purified enzyme showed that lowering the temperature results in a significant increase of the pure protein yield, similarly to the observation in whole cells, but also a relevant decrease in specific activity (Fig. 1D). The fact that in whole cells the total activity reached a maximum at 20°C is reasonably given by the combination of a very large

amount of protein with low activity; on the contrary, at 30°C the amount of protein is much lower, but the specific activity is higher, reaching 1880±108 μmol H_2/min/mg protein. Also, it is important to consider that the purified enzyme obtained by expression at 20°C formed aggregates when the concentration was increased, while the enzyme expressed either at 25°C or 30°C was readily soluble and could be concentrated by ultra-filtration up to the millimolar range.

The increase in specific activity and solubility at higher expression temperature is probably a result of improved protein folding, iron sulphur clusters incorporation and maturation (*i.e.* incorporation of the H-cluster catalytic centre). Even if the amount of the maturases CaHydF and CaHydG is lower at 30°C, this might represent the best molar ratios between the proteins, leading to optimal kinetics of the process of the metal centre assembly, and availability of the cellular substrates, such as iron and tyrosine, resulting in a high proportion of holo-CaHydA. Lowering the post-induction temperature is a common procedure in recombinant expression of proteins in *E. coli*, as it usually leads to slower kinetics hence avoiding the formation of inclusion bodies and improving recovery of the target protein [38,39]. Indeed in our case this effect was observed: the protein amount was larger at lower temperatures, but it did not correlate with specific activity, as this is the result of a more complex process, as discussed above. Another possible tuning effect of the temperature might involve endogenous *E. coli* scaffold proteins for iron-sulfur cluster biosynthesis, which must be recruited for hydrogenase assembly, either affecting the H-cluster or the other FeS clusters inserted in this enzyme [40,41]. For example, it was shown that the scaffold protein IscU from *Escherichia coli* has a tight temperature control with a narrow range of activity [42].

The protocol described here, with the expression at 30°C, resulted in the highest specific activity reported so far for the recombinant CaHydA. The H_2 evolution rate of 1880±108 μmol H_2/min/mg protein, assayed by gas chromatography with 10 mM reduced methyl

viologen as artificial electron donor, is in line with the specific activity of other recombinant [FeFe]-hydrogenases (Table I) and in the same order of magnitude of other native [FeFe]-hydrogenases from Clostridia [43-45].

These results may be very useful in the future to standardise the process and to simplify comparison between different enzyme preparations from different laboratories. Also, the effect of the temperature on specific activity of purified enzymes can contribute to explain the apparent incongruences previously reported in recent mutagenesis studies [11-14,18].

In conclusion, the results presented here show that the post-induction temperature has a relevant effect on the pure protein yield of CaHydA [FeFe]-hydrogenase and on the specific activity of a properly assembled H-cluster in the purified enzyme, with reverse proportionality. The maximum specific activity was observed when the post-induction temperature was 30°C. Despite the lower yield of pure protein, it is clear that the solubility and the higher specific activity, given by a higher proportion of holo-enzyme, are important factors for the characterisation of [FeFe]-hydrogenases and for their effective exploitation in future applications in biotechnology.

Materials and methods

Recombinant expression

The plasmids pCaE2 and pCaFG encoding for CaHydA and the maturases CaHydE, CaHydF and CaHydG [8] were co-transformed into *E. coli* Rosetta2(DE3). As previously described [10], bacteria were aerobically grown in baffled flasks (VWR) at 37°C in terrific broth (12 g/L tryptone, 24 g/L yeast extract, 4 mL/L glycerol, 2.2 g/L KH_2PO_4, 9.4 g/L K_2HPO_4) supplemented with 200 µg/mL carbenicillin, 50 µg/mL streptomycin, 34 µg/mL chloramphenicol and 2 mM ammonium ferric citrate. When the OD_{600} reached ~0.4, the

culture was supplemented with 2 mM cysteine, 25 mM fumarate, 0.5% w/v glucose and induced with 1.5 mM IPTG.

Immediately after induction, the culture was split in sterile glass vials (100 mL each), sealed and purged with pure argon to remove trace oxygen, allowing the expression of the active enzymes. The vials were then incubated 22 hours at different temperatures ranging from 4°C to 37°C.

Protein expression analysis

Total cell lisates were separated by SDS-PAGE on 10% polyacrylamide gels and stained with Coomassie R350 (GE Healthcare). Western blot against Strep-TagII was performed on PVDF membranes (GE Healthcare) with the Strep-Tactin HRP conjugate (IBA) and stained with 3,3'-diaminobenzidine (Sigma-Aldrich).

Enzyme purification

All the manipulations were carried out under strict anaerobic conditions in a glove box (Plas Labs) under a hydrogen-nitrogen atmosphere. All solutions were equilibrated with the glove box atmosphere and supplemented with 2-20 mM sodium dithionite before use.

CaHydA was purified by affinity chromatography by Strep-Tactin Superflow high capacity cartridges (IBA, Goettingen, Germany) as previously described [18].

Purified protein yield was determined with the Bradford assay using bovine serum albumin as standard (Sigma-Aldrich).

Activity assays

Hydrogenase activity (H_2 evolution) was determined at 37°C as previously described [18].

Briefly, reactions were set up in anaerobic 100 mM TrisHCl, 150 mM NaCl, pH 8.0 with 10

mM methyl viologen and 20 mM sodium dithionite. For the determination of the whole cells activity 0.1% v/v Triton X-100 was also added and the reaction was started by the addition of the culture. For the determination of the specific activity, the reactions were started by the addition of the purified enzyme.

H_2 evolution was quantified by gas chromatography, using an Agilent Technologies 7890A instrument equipped with purged packed inlet, Molesieve 5A column (30 m, ID 0.53 mm, film 25 mm) and thermal conductivity detector; argon was used as carrier gas.

Acknowledgements

This work was supported by "RICERCA LOCALE" 2012 and 2013 from the University of Torino and, partially, by project HyStrEM (E.U. Structural Funds N.1083/2006 F.E.S.R. 2007-2013). The authors declare no conflicts of interest.

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1 **Table and Caption.**

Type	Host	Maturases	Enzyme	Specific activity (µmol H_2/min/mg)	Yield (mg/L)	Ref.
Cell-Hosted	***E. coli*** **Rosetta2(DE3)**	**Ca**	**CaHydA**	**1880±108** **GC 10 mM MV pH 8.0**	**1.2**	**This work**
	E. coli Rosetta2(DE3)	Ca	Fd-CrHydA1	1000 GC 10 mM MV pH 8.0	5	[10]
	E. coli BL21(DE3) *ΔiscR*	So	CrHydA1	641±88 GC 5 mM MV pH 6.8	30±11	[20]
	S. oneidensis	endog.	CrHydA1	740±56 Electrode 5 mM MV pH 6.7	0.5	[31]
	C. acetobutylicum	endog.	CaHydA	1750* GC MV pH 6.8	0.8	[9]
		endog.	CrHydA1	625* GC MV pH 6.8	1	
	E. coli BL21(DE3)	Ca	CaHydA	75.2 GC 5 mM MV pH 7-8	NR	[8]
		Ca	CrHydA1	150 GC 5 mM MV pH 7-8	0.8-1.0	
Cell-Free	-	-	CpI	2037±616 GC 10 mM MV pH 6.8	NR	[37]
	-	Ca	CrHydA1	700-800 GC 10 mM MV pH 6.8	NR	[36]
	-	So	CpI	~700** Spect. MV	NR	[35]
	-	Ca	CsHydA	~2.5 GC 10 mM MV pH 7.5	NR	[32]

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Table I. Comparison of the specific activity and yield of CaHydA with other recombinant [FeFe]-hydrogenases. Ca) *Clostridium acetobutylicum*. Cr) *Chlamydomonas reinhardtii*. So) *Shewanella oneidensis*. Cs) *Clostridium saccharobutylicum*. endog.) endogenous maturases. Fd) ferredoxin. Without other specification, specific activity is reported as H_2 evolution rate. *) V_{max}. **) H_2 oxidation rate. The methodology used is also indicated: GC) Gas chromatography. Spect.) Spectrophotometric assay. MV) methyl viologen as artificial redox partner. The pH of the assay is also specified. Protein yield is reported as mg pure protein obtained per litre of culture. NR) not reported.

Figure Caption

Figure 1. (2-column fitting)

Effect of the post-induction temperature on the recombinant expression of CaHydA. A) Coomassie stained SDS-PAGE of whole cells lisates; bands at the molecular weight of CaHydF (46 kDa) and CaHydG (53 kDa) are marked. NI = Not induced. B) Western blot against Strep-tagII; a band at the molecular weight of CaHydA (65 kDa) can be identified. C) Total hydrogenase activity of whole cells. D) Specific activity of purified CaHydA (continuous line, filled squares) and yield of pure protein (dashed line, open squares).