FEBS Letters 438 (1998) 124–126 FEBS 21045

$F_{420}H_2$:NADP oxidoreductase from *Methanobacterium thermoautotrophicum*: identification of the encoding gene via functional overexpression in *Escherichia coli*

Holger Berk, Rudolf K. Thauer*

Max-Planck-Institut für terrestrische Mikrobiologie and Laboratorium für Mikrobiologie, Fachbereich Biologie der Philipps-Universität Marburg, Karl-von-Frisch-Straße, D-35043 Marburg, Germany

Received 15 September 1998

Abstract $F_{420}H_2$:NADP oxidoreductase is found in methanogenic, sulfate-reducing and halophilic archaea and also in some bacteria. The putative gene encoding the enzyme was cloned from *Methanobacterium thermoautotrophicum* (strain Marburg) and heterologously expressed in *Escherichia coli*. The overproduced active enzyme was purified, characterized and crystallized.

Key words: Coenzyme F_{420}; NADP-binding motif; F_{420}-dependent NADP reductase; Methanogenic archaeon; *Methanobacterium thermoautotrophicum*

1. Introduction

$F_{420}H_2$:NADP oxidoreductase catalyzes the reversible hydride transfer from $F_{420}H_2$ to $NADP^+$: $F_{420}H_2+NADP^+ = F_{420}+NADPH+H^+$ ($\Delta G^{\circ\prime} = -7.7$ kJ/mol). The enzyme has been purified from *Methanobacterium thermoautotrophicum* (strain ΔH) [1], *Methanococcus vannielii* [2], *Methanogenium organophilum* [3], *Archaeoglobus fulgidus* [4], *Halobacterium cutirubrum* [5] and *Streptomyces griseus* [6]. It is a dimeric or tetrameric enzyme composed of only one type of subunit of apparent molecular mass between 28 kDa and 43 kDa and devoid of a chromophoric prosthetic group. In agreement with the latter finding, the enzyme exhibits a ternary complex catalytic mechanism [1–6].

In most of the methanogenic and sulfate-reducing archaea the function of $F_{420}H_2$:NADP oxidoreductase is to catalyze NADP reduction for biosynthesis. In these organisms the enzyme is found only in very low amounts. In some alcohol fermenting methanogens, however, the enzyme is involved in the generation of $F_{420}H_2$ required for CO_2 reduction to methane [3]. The function in the other organisms is not yet known.

The N-terminal amino acid sequence of the $F_{420}H_2$:NADP oxidoreductase from *M. organophilum* has recently been determined [3]. It shows sequence similarity to the N-terminal sequence of putative proteins encoded by the open reading frames MTH 248, MJ 1501 and AF 0887 and AF 1209 in the completely sequenced genomes of *M. thermoautotrophicum* (strain ΔH) [7], *Methanococcus jannaschii* [8] and *A. fulgidus* [9], respectively. The sequence similarity indicates but does not prove that these genes encode $F_{420}H_2$:NADP oxidoreductase. It could be that the sequence similarity only reflects a common binding motif for NADP or F_{420} and that the mentioned open reading frames encode other NADP- or F_{420}-dependent enzymes. Indeed, the amino acid sequence of the putative gene product exhibits the sequence motif $GxGx_2Gx_3Ax_6Gx_7R$, very similar to a motif proposed to be involved in NADP binding [10,11].

We describe here the heterologous expression of the gene MTH 248 from *M. thermoautotrophicum* (strain Marburg) in *Escherichia coli* and show that the gene product catalyzes the reversible reduction of NADP with $F_{420}H_2$ with high specific activities. Crystals of the overproduced enzyme were obtained. The gene MTH 248 from *M. thermoautotrophicum* (Marburg) was designated *fno* (F_{420}, NADP, oxidoreductase). The nucleotide sequence has been submitted to the EMBL Nucleotide Sequence Data Base with the accession number Y17210.

2. Materials and methods

M. thermoautotrophicum strain Marburg DSM 2133 was from the Deutsche Sammlung für Mikroorganismen und Zellkulturen. The λ ZAP Express gene library was that described by Hochheimer [12]. The cloning vector λ ZAP Express (*Sau*3AI/CIAP treated), the helper phage ExAssist and *E. coli* strains XLOLR and XL1-Blue MRF′ were from Stratagene. The cloning vector pCR-Blunt was from Invitrogen and the expression vector pET17b and *E. coli* BL21 (DE3) were from Novagen. The DIG luminescent detection kit and the PCR DIG probe synthesis kit were from Boehringer Mannheim.

2.1. Cloning and sequencing of the *fno* gene

A homologous probe was generated by PCR using genomic DNA from *M. thermoautotrophicum* (Marburg) as template and oligonucleotides derived from the 5′ end of the MTH 248 gene from *M. thermoautotrophicum* (ΔH): ATGAAGATAGCAGTTATTGGTGGAAC (sense); CACCAGACAGTCACAGTCCACTGG (antisense). A 482 bp fragment was amplified and labelled with digoxigenin-UTP and the labelled 482 bp fragment used to screen the λ ZAP Express gene library of *M. thermoautotrophicum* (Marburg). From one of the positive clones, the phagemid pHB1 was generated by excision and recircularization of pBK-CMV carrying a 9 kbp *Sau*3A fragment with the *fno* gene which was sequenced using the dideoxynucleotide method of Sanger et al. [13].

2.2. Heterologous expression of the *fno* gene

The *fno* gene was amplified by PCR using pHB1 plasmid DNA as template and the oligonucleotides **CA**TATGAAGATCGCAGTTCTTGGTGGAAC (sense) and AAA**GGCCTT**TCCAACA**GGATCC**GACCTCCCTGTG (antisense) as primers. The sense oligonucleotide was derived from the 5′ DNA sequence of *fno* around the start codon ATG and contained a *Nde*I site (restriction site bases underlined; mutated bases in bold). The antisense oligonucleotide was derived downstream of the UGA stop codon and contained a *Bam*HI site (restriction site bases underlined; mutated bases in bold). The modified coding sequence was cloned via PCR Blunt into the expression vector pET17b. With the resulting plasmid pHB4, *E. coli* BL21 (DE3) was transformed. For expression of *fno* the transformed *E. coli* was grown anaerobically on Luria-Bertani medium containing ampicillin

*Corresponding author. Fax: (49) (6421) 178209.
E-mail: thauer@mailer.uni-marburg.de

PII: S0014-5793(98)01288-5

(100 μg/ml) to an absorbance value of 1.0 and subsequently induced by the addition of isopropylthio-β-D-galactopyranoside to 1 mM. After 3 h of further growth the cells were harvested by centrifugation.

2.3. Preparation of cell extract

The *E. coli* cells, transformed, grown and harvested as described above, were suspended in 50 mM Tris-HCl pH 7.6 to a concentration of 25 mg protein/ml. The suspension was passed three times through a French Press Cell at 150 MPa and then centrifuged for 45 min at 27 000×*g* at 4°C. The supernatant is designated cell extract.

3. Results and discussion

The *fno* gene encoding $F_{420}H_2$:NADP oxidoreductase was cloned from the Marburg strain of *M. thermoautotrophicum* rather than from the ΔH strain because most biochemical studies in recent years have been performed with the Marburg strain which grows more rapidly and to much higher cell densities than the ΔH strain [14]. The two strains, which both grow optimally at 65°C, are phylogenetically closely related [14,15]. This is also reflected in the sequence of the *fno* gene in the Marburg and ΔH strains. The two genes were found to show a high degree of sequence identity both at the DNA level (80%) and at the protein level (81%).

The *fno* gene in the Marburg strain encodes a putative protein composed of 224 amino acids with a molecular mass of 23.5 kDa and a p*I* of 4.6. It is preceded at a distance of 80 bp and followed at a distance of 25 bp by open reading frames encoding conserved proteins of unknown function. The gene upstream of *fno* shows a high degree of sequence identity with the gene MTH 247 and the gene downstream of *fno* with the gene MTH 249 in the completely sequenced genome of the ΔH strain [7].

The *fno* gene was heterologously expressed in *E. coli* and the gene product found to be overproduced after induction

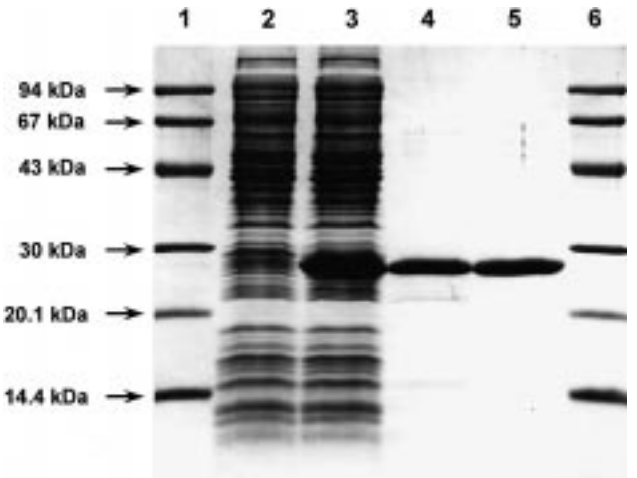

Fig. 1. Expression of the *fno* gene of *M. thermoautotrophicum* strain Marburg in *E. coli* BL21 (DE3) carrying pHB4 as evidenced by SDS-PAGE. Protein was separated on a 16% polyacrylamide gel, which was stained with Coomassie brilliant blue R250 [16]. Lanes 1 and 6, 5 μg molecular mass standards; lane 2, 20 μg cell extract protein of *E. coli* (pHB4) grown in the absence of isopropylthio-β-D-galactopyranoside (IPTG); lane 3, 25 μg cell extract protein of *E. coli* (pHB4) after induction with IPTG; lane 4, 4 μg of the 12 000×*g* supernatant of cell extract of IPTG-induced cells after heat treatment; lane 5, 4 μg of purified enzyme after chromatography on Resource Q.

(Fig. 1, lane 3). Cell extracts (16 mg protein/ml) of induced cells catalyzed the reduction of F_{420} (70 μM) with NADPH (1 mM) at pH 6.0 with a specific activity of 70 U/mg. The enzyme could be purified almost to homogeneity by simply heating the cell extract for 10 min at 70°C and by subsequent removal of precipitated protein by centrifugation (Fig. 1, lane 4). Via the heat step the specific activity increased to 180 U/mg. Remaining contaminants were removed by affinity chromatography on 2′,5′-ADP-agarose (eluted with 50 mM Tricine/KOH pH 8.0 containing 2 M KCl and 2 mM $NADP^+$) followed by anion exchange chromatography on Resource Q (eluted with 50 mM Tricine/KOH pH 8.0 containing 0–2 M KCl). The specific activity of the purified enzyme was 280 U/mg. The overproduced enzyme thus had to be purified only 4-fold. The purified enzyme at a protein concentration of 20 mg/ml was completely stable when stored at 4°C in 50 mM Tricine/KOH pH 8.0 containing 0.2 M KCl.

The purified enzyme was composed of only one type of subunit with an apparent molecular mass of 28 kDa as determined by SDS-PAGE. Gel filtration chromatography on Superdex 200 revealed an apparent molecular mass of 110 kDa suggesting that the overproduced enzyme is a homotetramer. The UV/visible spectrum was identical to that of bovine serum albumin. The pH optimum for F_{420} reduction (70 μM) with NADPH (1 mM) was found to be near 3.5 (640 U/mg) and for NADP reduction (1 mM) with $F_{420}H_2$ (0.1 mM) to be near 8.0 (830 U/mg). The K_m values were determined to be 0.3 mM for F_{420} (pH 6.0), 50 μM for NADPH (pH 6.0), 0.15 mM for $F_{420}H_2$ (pH 8.0) and 70 μM for $NADP^+$ (pH 8.0) and the respective V_{max} values were 1500 U/mg for F_{420} reduction with NADPH at pH 6.0 and 1100 U/mg for $NADP^+$ reduction with $F_{420}H_2$ at pH 8.0. The overproduced *fno* gene product thus showed almost the same molecular and catalytic properties as $F_{420}H_2$:NADP oxidoreductase purified from *M. thermoautotrophicum* (strain ΔH) [1]. The only difference observed was a 3-fold higher specific activity of the overproduced enzyme. This can be explained by the fact that the overproduced enzyme had to be purified only 4-fold whereas the enzyme from the ΔH strain had to be purified over 3400-fold during which some inactivation probably occurred.

$F_{420}H_2$:NADP oxidoreductase overproduced in *E. coli* crystallized in 100 mM sodium citrate pH 5.6 containing 40% isopropanol and 10% polyethylene glycol 4000 at 4°C employing the hanging drop method. The crystals grew within 1 week to a size of $0.02\times0.02\times0.2$ mm^3 but the size did not increase upon further incubation. The results show that the enzyme is susceptible to crystallization and thus in principle to X-ray structure analysis which will be the aim of future studies using the same methods previously employed for the elucidation of the crystal structure of formyltransferase [17,18] and methylcoenzyme M reductase [19,20] from methanogenic archaea. The finding that the *fno* gene from *M. thermoautotrophicum* can be functionally expressed in *E. coli* indicates that this is probably also possible for the respective gene from other organisms. Future studies thus will not be restricted to investigations of the enzyme from *M. thermoautotrophicum*.

Acknowledgements: This work was supported by the Max-Planck-Gesellschaft, by the Deutsche Forschungsgemeinschaft and by the Fonds der Chemischen Industrie.

References

[1] Eirich, L.D. and Dugger, R.S. (1984) Biochim. Biophys. Acta 802, 454–458.
[2] Yamazaki, S. and Tsai, L. (1980) J. Biol. Chem. 255, 6462–6465.
[3] Berk, H. and Thauer, R.K. (1997) Arch. Microbiol. 168, 396–402.
[4] Kunow, J., Schwörer, B., Stetter, K.O. and Thauer, R.K. (1993) Arch. Microbiol. 160, 199–205.
[5] De Wit, L.E.A. and Eker, A.P.M. (1987) FEMS Microbiol. Lett. 48, 121–125.
[6] Eker, A.P.M., Hessels, J.K.C. and Meerwaldt, R. (1989) Biochim. Biophys. Acta 990, 80–86.
[7] Smith, D.R., Doucette-Stamm, L.A., Deloughery, C., Lee, H., Dubois, J., Aldredge, T., Bashirzadeh, R., Blakely, D., Cook, R., Gilbert, K., Harrison, D., Hoang, L., Keagle, P., Lumm, W., Pothier, B., Qiu, D., Spadafora, R., Vicaire, R., Wang, Y., Wierzbowski, J., Gibson, R., Jiwani, N., Caruso, A., Bush, D., Safer, H., Patwell, D., Prabhakar, S., McDougall, S., Shimer, G., Goyal, A., Pietrokovski, S., Church, G.M., Daniels, C.J., Mao, J.I., Rice, P., Nölling, J. and Reeve, J.N. (1997) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
[8] Bult, C.J., White, O., Olsen, G.J., Zhou, L., Fleischmann, R.D., Sutton, G.G., Blake, J.A., FitzGerald, L.M., Clayton, R.A., Gocayne, J.D., Kerlavage, A.R., Dougherty, B.A., Tomb, J.F., Adams, M.D., Reich, C.I., Overbeek, R., Kirkness, E.F., Weinstock, K.G., Merrik, J.M., Glodek, A., Scott, J.L., Geoghagen, N.S.M., Weidman, J.F., Fuhrmann, J.L., Nguyen, D., Utterback, T.R., Kelley, J.M., Peterson, J.D., Sadow, P.W., Hanna, M.C., Cotton, M.D., Roberts, K.M., Hurst, M.A., Kaine, P.P., Borodovsky, M., Klenk, H.P., Fraser, C.M., Smith, H.O., Woese, C.R. and Venter, J.C. (1996) Science 273, 1058–1073.
[9] Klenk, H.-P., Clayton, R.A., Tomb, J.-F., White, O., Nelson, K.E., Ketchum, K.A., Dodson, R.J., Gwinn, M., Hickey, E.K., Peterson, J.D., Richardson, D.L., Kerlavage, A.R., Graham, D.E., Kyrpides, N.C., Fleischmann, R.D., Quackenbush, J., Lee, N.H., Sutton, G.G., Gill, S., Kirkness, E.F., Dougherty, B.A., McKenney, K., Adams, M.D., Loftus, B., Peterson, S., Reich, C.I., McNeil, L.K., Badger, J.H., Glodeck, A., Zhou, L., Overbeek, R., Gocayne, J.D., Weidman, J.F., McDonald, L., Utterback, T., Cotton, M.D., Spriggs, T., Artiach, P., Kaine, B.P., Sykes, S.M., Sadow, P.W., D'Andrea, K.P., Bowman, C., Fujii, C., Garland, S.A., Mason, T.M., Olsen, G.J., Fraser, C.M., Smith, H.O., Woese, C.R. and Venter, J.C. (1997) Nature 390, 364–370.
[10] Scrutton, N.S., Berry, A. and Perham, R.N. (1990) Nature 343, 38–43.
[11] Hanukoglu, I. and Gutfinger, T. (1989) Eur. J. Biochem. 180, 479–484.
[12] Hochheimer, A. (1997) Doctoral Thesis, Philipps-Universität, Marburg.
[13] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
[14] Brandis, A., Thauer, R.K. and Stetter, K.O. (1981) Zbl. Bakt. Hyg. I Abt. Orig. C 2, 311–317.
[15] Nölling, J., Hahn, D., Ludwig, W. and de Vos, W.M. (1993) Syst. Appl. Microbiol. 16, 208–215.
[16] Laemmli, U.K. (1970) Nature 227, 680–685.
[17] Shima, S., Thauer, R.K., Michel, H. and Ermler, U. (1996) Proteins Struct. Func. Genet. 26, 118–120.
[18] Ermler, U., Merckel, M.C., Thauer, R.K. and Shima, S. (1997) Structure 5, 635–646.
[19] Shima, S., Goubeaud, M., Vinzenz, D., Thauer, R.K. and Ermler, U. (1997) J. Biochem. 121, 829–830.
[20] Ermler, U., Grabarse, W., Shima, S., Goubeaud, M. and Thauer, R.K. (1997) Science 278, 1457–1462.