FEBS 19217 FEBS Letters 414 (1997) 545–548

Purification and characterization of a flavohemoglobin from the denitrifying fungus *Fusarium oxysporum*

Naoki Takaya, Sawako Suzuki, Masaru Matsuo, Hirofumi Shoun*

Institute of Applied Biochemistry, University of Tsukuba, Tsukuba, Ibaraki 305, Japan

Received 14 July 1997; revised version received 12 August 1997

Abstract A flavohemoprotein was purified to homogeneity from the denitrifying fungus *Fusarium oxysporum*. The purified protein existed as a monomer with a molecular weight of 44 kDa. It was purified in an oxidized form and exhibited the absorption maxima at 401, 540 and 643 nm in its resting form, and at 434 and 555 nm upon reduction with dithionite, respectively. The protein contained 0.5 mol protoheme/mol and 1.1 mol FAD/mol, respectively. When the resting flavohemoprotein was aerobically incubated with NAD(P)H, it was converted to a spectral species that is spectrally very similar to oxyhemoglobins. These properties are characteristics of flavohemoglobins (FHb) of *Alcaligenes eutrophus*, *Escherichia coli*, and baker's yeast. Further the amino terminal amino acid sequence of the protein of *F. oxysporum* was similar to those of these FHbs. These results suggest that the isolated flavohemoprotein of *F. oxysporum* would be a counterpart of the proteins in the FHb family.

Key words: Flavohemoprotein; Hemoglobin; O_2 binding; NAD(P)H oxidase; Denitrification; *Fusarium oxysporum*

1. Introduction

Denitrification is an anaerobic respiratory system previously found only in bacteria and was thought to be a unique character for prokaryotes. Recently, however, we revealed that many filamentous fungi have denitrifying activities for the first evidence of denitrification by eukaryotes [1,2]. As in the bacterial cases the fungal denitrification acts as anaerobic (nitrate) respiration, and the system was shown to be localized into mitochondria [3,4]. Bacterial denitrifying systems have been shown to consist of many components. So it is of particular interest and importance to isolate and characterize the components of fungal systems as many as possible and to compare each component with the bacterial counterpart. We have been isolating and analyzing several components involved in the fungal systems of *Fusarium oxysporum* and *Cylindrocarpon tonkinense* such as nitrate reductase, nitrite reductase and its electron transferring proteins [3], and nitric oxide reductases [5–7].

Flavohemoglobins (FHbs) are found in both prokaryotes and eukaryotes such as *Alcaligenes eutrophus* [8–10], *Escherichia coli* [11,12], *Saccharomyces cerevisiae* [13] and *Candida norvegensis* [14]. They have FAD and protoheme and the spectral properties of the heme moiety are quite similar to those of hemoglobins and myoglobins as well as their oxygen binding ability. They receive electrons from NAD(P)H in the presence of oxygen, and it is suggested that it can reduce oxygen to generate superoxide in case of *E. coli* FHb [15]. Their physiological roles, however, have not been cleared yet. FHb in *A. eutrophus* is proposed to function in anaerobic growth as the mutant fails to accumulate nitrous oxide under denitrifying condition [9]. On the contrary, *S. cerevisiae* FHb is suggested to be involved in oxidative stress response, because the expression of FHb is induced by oxidative stress [16] and the growth of its mutant is substantially sensitive against reagents that promote oxidative stress [17]. It is proposed that *E. coli* FHb acts as an oxygen sensor on the basis of the fact that redox state of the FAD moiety varies depending on environmental oxygen concentration [18].

Throughout the course of studies on fungal denitrification, we have isolated several components involved in the denitrification [3,5,7], all of which are metalloproteins. Recently, we found another hemoprotein in the cells of *F. oxysporum* grown under denitrifying conditions. In this study, we isolated the hemoprotein and characterized its properties, and suggested that it is a counterpart of the FHbs. We further discussed on the possible relationship of the fungal FHb to denitrification.

2. Materials and methods

2.1. Strain, culture, and media

F. oxysporum MT-811 first identified as a eukaryotic denitrifier [1] was cultivated as described previously [3], in a 5 l Erlenmeyer flask with a cotton plug containing 3 l of the medium that consist of 3% glycerol, 0.2% soybean flour, 10 mM nitrate, and trace elements [3]. Incubation was done on a rotary shaker at 130 rpm for the first 30 h, at 110 rpm for the following 18 h, and then the flask was stood without shaking for the last 24 h. All steps were done at 30°C. This incubating condition results in inducing the best denitrifying activity in *F. oxysporum* [3,19].

2.2. Purification of flavohemoprotein

All procedures were performed below 4°C except fast protein liquid chromatography (FPLC) that was performed at room temperature. Approximately 100 g of the cells were disrupted by grinding with alumina as reported [2] and centrifuged at $10000 \times g$ for 20 min. The resultant supernatant was dialyzed against buffer A (20 mM KH_2PO_4-NaOH (pH 7.4), 10% glycerol, 0.1 mM EDTA, 0.1 mM dithiothreitol), and then subjected to following chromatographies where the hemoproteins were monitored by the absorbance at 405 nm. The sample was applied to a DEAE-cellulose column (Whatman DE52; 25 mm diameter × 50 mm length) equilibrated with buffer A. After washing, proteins were eluted by 0–500 mM NaCl gradient in buffer A. Heme containing fraction was collected and dialyzed against 50 mM sodium citrate buffer (pH 5.5) containing 0.1 mM EDTA and 0.1 mM dithiothreitol, and applied to a Hi-Trap SP column (Pharmacia) equilibrated with the same buffer. Flow through fraction was dialyzed against buffer A and applied to an FPLC instrument (Pharmacia) equipped with a MonoQ HR5/5 column equilibrated with buffer A. A deep brown colored fraction was eluted by 0–300 mM NaCl gradient in the same buffer, which was collected and directly

*Corresponding author. Fax: +81 (298) 53-4605.
E-mail: p450nor@sakura.cc.tsukuba.ac.jp

PII S0014-5793(97)01069-7

applied to a Superdex 200HR 10/30 column (Pharmacia) equilibrated with 50 mM sodium phosphate (pH 7.4) containing 150 mM NaCl. The sample after this step was used as the purified FHb.

2.3. Analytical methods

Optical and fluorescence spectra were measured with a Beckman DU 7500 spectrophotometer and a Hitachi F-3010 fluorescence spectrophotometer, respectively. Heme was identified and determined by the pyridine ferrohemochromogen method employing the molar absorption coefficient (ε) of the chromogen of the protoheme as 34.4 mM^{-1} cm^{-1} at 557 nm [20]. The FAD content was estimated using $\varepsilon = 11.5$ mM^{-1} cm^{-1} at 450 nm [21]. Protein concentration was determined with Bio-Rad protein assay reagent according to the manufacturer's instruction. Amino terminal amino acid sequence was determined with a Perkin Elmer Procise 492 automated protein sequencer.

3. Results and discussion

3.1. Purification of flavohemoprotein

During the routine works for purifying P450nor [5], one of the major hemoproteins in the crude extracts from denitrifying cells of *F. oxysporum*, we noticed that a lot of heme-like pigments remains on the first DEAE-cellulose column after eluting P450nor. This time, we purified the pigment as described in Section 2 and the result is summarized in Table 1. As the purified protein bound carbon monoxide (CO) efficiently (see Section 3.3), it was quantified from the difference spectrum of (reduced plus CO) minus (reduced) employing ε for the difference between 420 and 437 nm to be 266.2 mM^{-1}, which was determined with respect to the purified preparation. The recovery (or total FHb) increased after the DEAE-cellulose column. This might depend on removal of P450nor, the CO-difference spectrum of which exhibits a minus value around 420 nm, so the amount of FHb in the crude extract would be underestimated. The purified protein gave a single band on SDS-PAGE and its molecular mass was estimated to be 44 kDa (Fig. 1A). The molecular mass estimated by gel filtration with a Superdex 200HR 10/30 column was 44 kDa (Fig. 1B), suggesting that the protein was present as a monomer. Although the starting crude extract would contain microsomes, the purified FHb must be a soluble protein because we could purify it without solubilizing treatment.

3.2. Prosthetic groups

As the isolated protein exhibited a clear Soret peak at 401 nm, we prepared its pyridine ferrohemochromogen. The resulting chromogen exhibited α-band at 557 nm, indicating that the heme prosthetic group was protoheme. The protein also exhibited a fluorescence. The emission spectrum (excited at 460 nm) showed its peak at 530 nm with a shoulder at 546 nm (data not shown), indicating that the hemoprotein also contained flavin cofactor. From these results and the fact that the flavohemoprotein has oxygen-binding ability (see Section 3.3), we designate the protein FHb, an abbreviation of

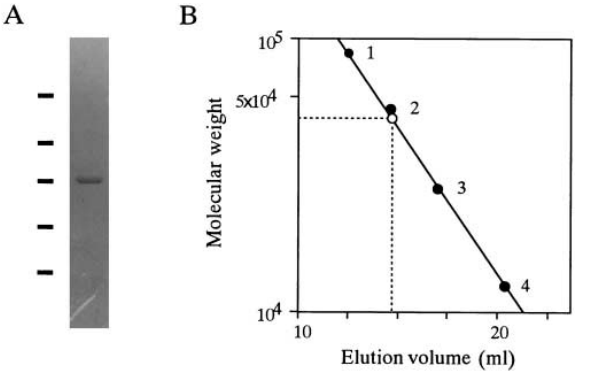

Fig. 1. Homogeneity and molecular weight determination of the flavohemoglobin. A: SDS-PAGE of purified FHb. Position of molecular weight markers (phosphorylase b, 94 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; soybean trypsin inhibitor, 20.1 kDa) are shown on the left. B: Gel filtration of FHb by Superdex 200HR 10/30 column. Open and closed circles represent elution volume of FHb and molecular weight marker, respectively. The molecular weight markers used are: 1, bovine serum albumin (67 kDa); 2, ovalbumin (43 kDa); 3, carbonic anhydrase (30 kDa); 4, soybean trypsin inhibitor (20.1 kDa).

flavohemoglobin. The emission peak was bleached upon addition of 0.4 mM NAD(P)H, indicating that the flavin would be reduced by NAD(P)H (data not shown). A similar phenomenon has been observed with *E. coli* flavohemoglobin [16]. The type of flavin was analyzed by TLC using FMN, FAD, and riboflavin as standards and identified to be FAD (data not shown). Contents of protoheme and FAD in the purified preparation were estimated as 0.5 mol/mol protein and 1.1 mol/mol protein, respectively. Heme would be partially dissociated from the FHb protein during the purification procedure. The lower heme content as compared with the FAD content is also observed in the case of other FHbs [11].

3.3. Spectral properties

F. oxysporum FHb was purified in an oxidized form and its absorption spectrum was characterized by a Soret peak at 401 nm, and β- and α-bands at 540 and 643 nm, respectively (Fig. 2). The peak at 484 nm would be attributed to high spin state of the heme iron. The dithionite-reduced (ferrous) form showed a Soret peak at 434 nm and a broad β-band at 555 nm. CO could be ligated to the dithionite-reduced FHb and gave the absorption maxima at 420, 539, and 567 nm. These spectral properties with respect to the heme moiety were similar to those of hemoglobins. The difference spectrum of the CO-bound minus dithionite-reduced forms showed a peak at 420 nm and a trough at 437 nm (data not shown).

On incubation with 1 mM NADH in aerated solution, the FHb altered into a spectral species with the absorption peaks at 415, 544, and 560 nm (Fig. 2), that is spectrally very similar to oxyhemoglobins, indicating that FHb could be reduced

Table 1
Purification of *F. oxysporum* flavohemoglobin

Purification step	Total protein (mg)	Total FHb (nmol)	Specific content (nmol/mg)	FHb recovery (%)	Purification fold
Crude extract	578	219.7	0.38	100	–
DEAE-cellulose	79.5	272.1	3.43	124	9.0
SP-Sepharose	61.9	240.2	3.88	109	10.2
MonoQ HR5/5	7.9	56.8	7.19	26	18.9
Superdex 200HR	3.7	39.1	10.57	18	27.8

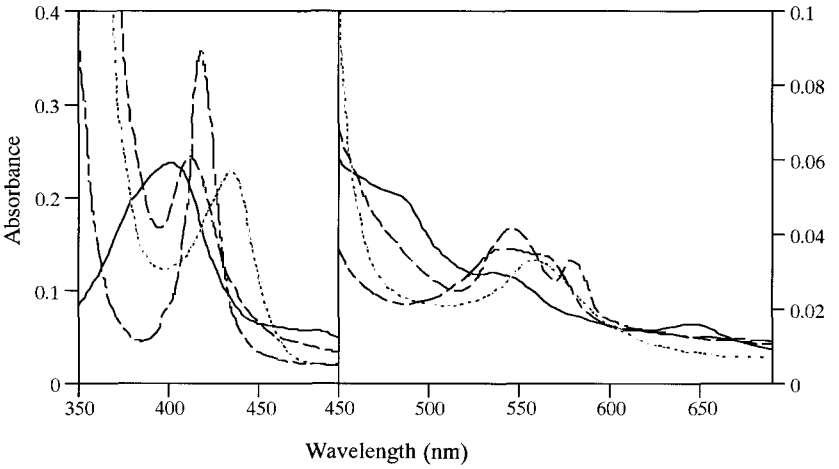

Fig. 2. Absorption spectra of the flavohemoglobin. Absorption spectra of FHb (0.8 μM in 50 mM sodium phosphate buffer (pH 7.2)) were measured at room temperature. Solid line, resting (oxidized); dotted line, dithionite-reduced; dashed line, NADH-reduced (1 mM NADH was added); dash-dotted line, dithionite-reduced plus CO.

with NADH to form an oxygenated species. The oxygenated species was gradually decomposed into the initial oxidized species after standing for several hours (Fig. 3). Incubation with the same concentration of NADPH gave the same result. It has been suggested that *E. coli* FHb has an NAD(P)H oxidase activity to reduce oxygen into superoxide anion [13]. Judging from the above results, it is possible that *F. oxysporum* FHb has the same activity. This was supported by the result that FHb showed an NAD(P)H-dependent cytochrome *c* reductase activity that was inhibited by superoxide dismutase (data not shown). This result can be interpreted that *F. oxysporum* FHb could oxidize NAD(P)H to reduce cytochrome *c* by producing superoxide anion as a catalytic intermediate. Physiological significance of this activity is to be elucidated.

3.4. Amino terminal amino acid sequence

The amino terminal amino acid sequence of FHb was determined. It showed significant similarity to those of FHbs from *A. eutrophus*, *S. cerevisiae*, and *E. coli* with identities between 30 and 55% (Fig. 4). From these sequence similarities and the spectral and the enzymatic properties described above, we concluded that FHb purified in this study is a member of the FHb family.

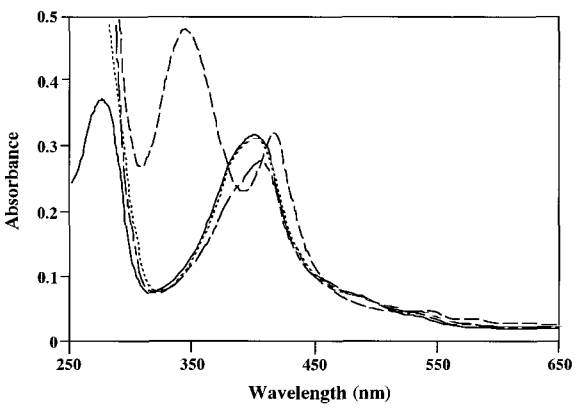

Fig. 3. Spectral changes of the flavohemoglobin upon incubation with NADH. FHb (0.8 μM) in 50 mM sodium phosphate buffer (pH 7.2) was mixed with 0.2 mM NADH under aerobic condition. Each spectrum shows: solid line, resting (oxidized); dashed line, at 10 s after addition; dash-dotted line, after 60 min; dotted line, after 300 min.

3.5. Concluding remarks

Although *F. oxysporum* is a eukaryote, the amino terminal amino acid sequence of *F. oxysporum* FHb is more similar to those of bacterial FHbs than that of yeast FHb, especially similar to that of the denitrifying bacterium *A. eutrophus*. Whereas the real physiological function(s) of the FHbs is not understood yet, Cramm et al. suggested that *A. eutrophus* FHb could participate directly or indirectly in the nitrous oxide evolution during denitrification although the purified FHb has no nitric oxide reductase (nitrous oxide generating) activity [7]. Evidence that both *F. oxysporum* and *A. eutrophus* have denitrifying activity and share the FHb proteins whose sequences are evolutionary conserved to each other suggests some common roles of the FHbs of these organisms in denitrification although the mechanism(s) is to be elucidated. On the contrary, it is unlikely that *F. oxysporum* FHb functions for the oxidative stress response as suggested in case of *S. cerevisiae* FHb [16,17], because oxygen supply is restricted under the culture condition employed in this study. Further studies on such as expression and mutation of FHb will reveal the physiological function of FHb in *F. oxysporum*.

```
                 ••      •••  •••  •  ••
FFhp     ALTAAQVAIVKSTAPILKEH
AFhp     MLTQKTKDIVKATAPVLAEH
YHb      MLAEKTRSIIKATVPVLEQQ
Hmp      MLDAQIIATVKATIPLLVET
          *          *  *  *  *
```

Fig. 4. Alignment of the amino terminal amino acid sequences of flavohemoglobins. Twenty amino acid sequences on the amino termina are compared. FFHb, AFhp, YHb, and Hmp indicate flavohemoglobins from *F. oxysporum*, *A. eutrophus* [9], *S. cerevisiae* [13], and *E. coli* [12], respectively. Identical residues are indicated by asterisks. Conserved residues between *F. oxysporum* FHb and *A. eutropus* FHb (AFhp) are marked by dots on the sequences.

Acknowledgements: This study was supported by Sakabe Project of TARA of University of Tsukuba and a Grant in Aid for Scientific Research from the Ministry of Education, Science, Culture of Japan.

References

[1] Shoun, H. and Tanimoto, T. (1991) J. Biol. Chem. 266, 11078–11082.
[2] Shoun, H., Kim, D.-H., Uchiyama, H. and Sugiyama, J. (1992) FEMS Microbiol. Lett. 94, 277–282.
[3] Kobayashi, M. and Shoun, H. (1995) J. Biol. Chem. 270, 4146–4151.
[4] Kobayashi, M., Matsuo, Y., Takimoto, A., Suzuki, S., Maruo, F. and Shoun, H. (1996) J. Biol. Chem. 271, 16263–16267.
[5] Nakahara, K., Tanimoto, T., Hatano, K., Usuda, K. and Shoun, H. (1993) J. Biol. Chem. 268, 8350–8355.
[6] Kizawa, H., Tomura, D., Oda, M., Fukamizu, A., Hoshino, T., Gotoh, O., Yasui, T. and Shoun, H. (1991) J. Biol. Chem. 266, 10632–10637.
[7] Usuda, K., Toritsuka, N., Matsuo, Y., Kim, D.-H. and Shoun, H. (1995) Appl. Environ. Microbiol. 61, 883–889.
[8] Probst, I., Wolf, G. and Schlegel, H.G. (1979) Biochim. Biophys. Acta 576, 471–478.
[9] Cramm, R., Siddiqui, R.A. and Friedrich, B. (1994) J. Biol. Chem. 269, 7349–7354.
[10] Ermler, U., Siddiqui, R.A., Cramm, R. and Friedrich, B. (1995) EMBO J. 14, 6067–6077.
[11] Ioannidis, N., Cooper, C.E. and Poole, R.K. (1992) Biochem. J. 1992, 649–655.
[12] Vasudevan, S.G., Armarego, W.L.F., Shaw, D.C., Lilley, P.E., Dixon, N.E. and Poole, R.K. (1991) Mol. Gen. Genet. 226, 49–58.
[13] Zhu, H. and Riggs, A.F. (1992) Proc. Natl. Acad. Sci. USA 89, 5015–5019.
[14] Iwaasa, H., Takagi, T. and Shikama, K. (1992) J. Mol. Biol. 227, 948–954.
[15] Membrillo-Hernández, J., Ioannidis, N. and Poole, R.K. (1996) FEBS Lett. 382, 141–144.
[16] Zhao, X.-J., Raitt, D., Burke, P.V., Clewell, A.S., Kwast, K.E. and Poyton, R.O. (1996) J. Biol. Chem. 271, 25131–25138.
[17] Crawford, M.J., Sherman, D.R. and Goldberg, D.E. (1996) J. Biol. Chem. 270, 6991–6996.
[18] Poole, R.K., Ioannidis, N. and Orii, Y. (1994) Proc. R. Soc. Lond. B 255, 251–258.
[19] Tomura, D., Obika, K., Fukamizu, A. and Shoun, H. (1994) J. Biochem. 116, 88–94.
[20] Falk, J.E. (1964) Porphyrins and Metalloporphyrins, p. 240, Elsevier, Amsterdam.
[21] Yagi, K. (1962) in: Methods of Biochemical Analysis (Glick, D., ed.), pp. 319–356, Willey, New York.