Conversion of Flavodoxin from Holoenzyme to Apoprotein during Growth Phase Changes in Helicobacter pylori

Hirofumi Shimomura,1* Shunji Hayashi,1 Kenji Yokota,2 Keiji Oguma,2 and Yoshikazu Hirai1

Division of Bacteriology, Department of Infection and Immunity, Jichi Medical University, Tochigi 329-0498, and Department of Bacteriology, Graduate School of Medicine, Dentistry and Pharmacology, Okayama University, Okayama 700-8558, Japan

Received 19 February 2007/Accepted 12 April 2007

The catabolic pathway for flavodoxin has yet to be clarified for any bacterial species. In this study, we found that the flavin mononucleotide in the flavodoxin of Helicobacter pylori is degraded to riboflavin via the phosphonomonoesterase activity of class C acid phosphatase. The result is a conversion of holoflavodoxin to apoflavodoxin.

Flavodoxin (FldA) is a small electron transferase that contains flavin mononucleotide (FMN) as a cofactor. FldA plays an important role in the electron transport system and is essential for bacterial survival (1, 2). In Helicobacter pylori, a pathogen responsible for chronic gastritis and peptic ulcer (4, 6, 12), FldA functions as an electron acceptor for pyruvate: flavodoxin oxidoreductase in the biosynthesis of acetyl-coenzyme A (5). In Escherichia coli, FldA plays an essential role in the 2-C-methyl-d-erythritol 4-phosphate pathway for isoprenoid biosynthesis (7). In Bacillus subtilis, nitric-oxide synthase has recently been demonstrated to function as an electron acceptor for YkuN, a flavodoxin of B. subtilis (11). No catabolic pathway for FldA, however, has been identified for any bacterial species.

In this study, we have identified a qualitative alteration of FldA in H. pylori: FMN-bound FldA holoenzyme (holo-FldA) is converted to FMN-free FldA apoprotein (apo-FldA). The decrease in holo-FldA levels appears to correlate with the accumulation of apo-FldA levels during long-term culture. We have also managed to purify and identify the agent involved in the conversion from holo- to apo-FldA by using the class C acid phosphatase (HppA), a nonspecific acid phosphatase (10). This report is the first to describe the role of HppA in FldA catabolism.

H. pylori NCTC 11638 was used as the bacterial strain for this study. The organisms were cultured with brain heart infusion broth containing 5% horse serum in an atmosphere of 5% O2, 10% CO2, and 85% N2 at 37°C but not in the incubations at 4°C and 25°C. The concentration of holo-FldA was converted to apo-FldA in the incubation at 4°C, whereas only the former was detected in the Px protein, whereas only the former was detected in the Px protein.

Cell lysates obtained from organisms cultured for 3 days and 14 days yielded Py protein and Px protein by anion-exchange chromatography with a Q-Sepharose column (Sigma-Aldrich Co., St. Louis, MO) equilibrated with 50 mM Tris buffer (pH 7.5). The purified Px and Py proteins were then identified as flavodoxin (FldA) by peptide mass fingerprinting (PMF) analysis (Shimadzu Co., Kyoto, Japan). Both matched the FldA protein (calculated pI value, 4.45) of H. pylori strain 26695, with a top score of 95 in the Mascot Search (Matrix Science Ltd., London, United Kingdom).

To characterize the two forms of FldA (Px and Py) in detail, the purified Px and Py proteins were then analyzed by matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI-TOF MS) (Shimadzu Co.). The mass spectrum of an FldA protein with a molecular mass of about 17.5 kDa and the mass spectrum of FMN (identified with the mass spectrum of an FldA protein with a molecular mass of about 17.5 kDa and the mass spectrum of FMN) were both detected in the Py protein, whereas only the former was detected in the Px protein (Fig. 2A). Thus, the Px protein was determined not to contain FMN. When the gel was irradiated by UV light at a 254-nm wavelength after native PAGE of the purified Py and Px proteins, a fluorescent yellow band of FMN was detected at the position corresponding to the Py protein band stained by CBB but not at the position corresponding to the Px protein band (Fig. 2B). The Py protein was thus confirmed to contain FMN. These results indicate that the fast-migrating FldA (Py protein) is a holoenzyme (holo-FldA) containing FMN, while the slow-migrating FldA (Px protein) is an FMN-free apoprotein (apo-FldA).

When the cell lysates obtained from the organisms cultured for 0.5 days were incubated overnight at 4°C, 25°C, and 37°C, the holo-FldA was converted to apo-FldA in the incubation at 37°C but not in the incubations at 4°C and 25°C. The conver-

* Corresponding author. Mailing address: Division of Bacteriology, Department of Infection and Immunity, Jichi Medical University, 3311-1 Yakushiji, Shimotsuke-shi, Tochigi 329-0498, Japan. Phone: 81-285-58-7332. Fax: 81-285-44-1175. E-mail: shimo@jichi.ac.jp.

Published ahead of print on 20 April 2007.
sion of holo-FldA to apo-FldA was apparently induced via an enzymatic activity, and the growing cells apparently were just as active as the aging cells in inducing the conversion, even though apo-FldA was rarely detected in cell lysates obtained from the growing cells on day 0.5 of the culture (Fig. 1B). These results suggest that *H. pylori* is endowed with some mechanism for the conversion of holo-FldA to apo-FldA as a catabolic process. Further investigations are required to clarify the regulation system controlling the accumulation of apo-FldA in growing cells.

The following experiments were carried out to examine the difference in migration between holo- and apo-FldA in native PAGE. The fluorescence intensity of FMN was scanned at an excitation wavelength of 536 nm, and the absorption bands of FMN were detected in purified holo-FldA with three peaks of the absorption curve, which was identical to the absorption curve of an authentic FMN (Fig. 2C, peaks 1 and 2). The FMN absorption bands disappeared when the purified holo-FldA was treated with dithiothreitol (DTT) at a final concentration of 50 mM, leaving only one weakly detectable FMN absorption band at the short wavelength (Fig. 2C, peak 3). This meant that the FMN in the purified holo-FldA was in an oxidized form and was reducible by DTT treatment. The FMN absorption bands were undetectable in the purified apo-FldA and in the holo-FldA from which FMN had been removed by 10% trichloroacetic acid (TCA) precipitation. These results confirm the absence of FMN in the purified apo-FldA and TCA-treated holo-FldA (an artifact apo-FldA). The native PAGE profiles of FldA were analyzed with purified holo-FldA (containing oxidized FMN), DTT-treated holo-FldA (containing reduced FMN), purified apo-FldA (without FMN), and TCA-treated holo-FldA (without FMN). The electrophoretic position of DTT-treated holo-FldA corresponded with that of the holo-FldA containing oxidized FMN. Thus, the reduction of the isoalloxazine ring in the FMN with DTT treatment did not influence the migration of holo-FldA in native PAGE. In contrast, the TCA-treated holo-FldA (an artifact apo-FldA) had been electrophoretically switched to a position identical to the position of the purified apo-FldA. These results confirmed the utility of native PAGE in analyzing the conversion from holo- to apo-FldA and the conversion from apo- to holo-FldA.

Holoflavodoxin of *Chondrus crispus*, a macroalga, has also

![FIG. 1. Alterations in CFU during microaerobic culture and native PAGE profiles of bacterial proteins. (A) *H. pylori* NCTC 11638 was cultured in brain heart infusion broth containing 5% horse serum in a microaerobic chamber. Numbers of CFU at the indicated culture times were determined by the conventional method. (B) Proteins (80 μg lane) in cell lysates prepared from organisms recovered at the indicated culture times were analyzed by native PAGE followed by CBB staining.](http://jb.asm.org/pacs/189/07/n4961f1a.jpg)

![FIG. 2. Analysis of holo-FldA (Py protein) and apo-FldA (Px protein). (A) Purified Py protein (upper), purified Px protein (middle), and an authentic FMN (lower) were subjected to MALDI-TOF MS with α-cyano-4-hydroxycinnamic acid used as a matrix. (B) After subjection of the Py (10 μg/lane) and Px (10 μg/lane) proteins to native PAGE, the proteins in the gel (lanes 1, Py; lanes 2, Px) were exposed to UV light at a 254-nm wavelength (right) and stained by CBB (left). (C) The fluorescence intensity of FMN was assayed with an authentic FMN (15 μg/ml, peak 1), purified holo-FldA (500 μg/ml, peak 2), and DTT-treated holo-FldA (500 μg/ml, peak 3) by scanning at an excitation wavelength of 536 nm.](http://jb.asm.org/pacs/189/07/n4961f2.jpg)
been found to migrate toward the positive pole faster than apoflavodoxin in native PAGE (9). The electrophoretic behaviors of holo- and apo-FldA in *H. pylori* and *C. crispus* were thus found to be compatible with those of two forms of flavodoxin in *C. crispus* in native PAGE. These results suggest that holo-FldA carries a stronger negative electric charge than does apo-FldA because of the phosphate group of FMN in the former. It is necessary, however, to carry out further investigations to clarify the differences in migration between holo- and apo-FldA in the native PAGE gel.

The conversion from apo- to holo-FldA was examined by native PAGE analysis. A purified apo-FldA or an artifact apo-FldA (prepared by TCA precipitation of a purified holo-FldA) was incubated in the presence or absence of FMN for 2 h at room temperature in 150 mM Tris buffer (pH 7.5). After incubation, the specimens were subjected to native PAGE with a control holo-FldA. Native PAGE analysis detected binding of FMN to the purified apo-FldA and the artifact apo-FldA: the apo-FldA incubated with FMN migrated toward the positive pole faster than the apo-FldA incubated without FMN in the gel, and the electrophoretic position of the apo-FldA with FMN was identical to the position of the control holo-FldA. These results indicate that apo-FldA spontaneously joins with FMN and thereby converts to holo-FldA.

As described above, some enzymatic activity was required for the conversion from holo-FldA to apo-FldA. To identify one of the activities involved in the conversion from holo-FldA to apo-FldA, we conducted experiments using cell lysates obtained from organisms cultured for 2 days (10^8.0 CFU/ml) and prepared with 50 mM Tris buffer (pH 7.5 or 8.6). In brief, cell lysates of pH 7.5 and 8.6 were applied to a Q-Sepharose (Sigma-Aldrich Co.) anion-exchange column and an SP Sepharose (Amersham Biosciences AB, Uppsala, Sweden) cation-exchange column, respectively, for fractionation with various concentrations of NaCl. After dialysis against 50 mM Tris buffer (pH 7.5), each fraction obtained from anion- or cation-exchange chromatography was incubated with purified holo-FldA overnight at 37°C in 50 mM Tris buffer (pH 7.5). The activity involved in the conversion from holo-FldA to apo-FldA was confirmed by monitoring the generation of apo-FldA from holo-FldA in native PAGE after the incubation. A sodium dodecyl sulfate-PAGE analysis revealed a protein with a molecular mass of 28 kDa at significant levels in the fractions exhibiting the activity involved in the conversion from holo-FldA to apo-FldA, though the expression levels of the same protein with a molecular mass of 28 kDa were lower than those of the activity involved in the conversion from holo-FldA to apo-FldA. PMF analysis (Shimadzu Co.) identified a 28-kDa protein (the activity involved in the conversion from holo-FldA to apo-FldA) with class C acid phosphatase from holo-FldA to apo-FldA via the phosphomonoesterase activity of HppA. (A) Holo-FldA (1 μg) was incubated in the presence or absence of HppA (0.05 μg) overnight at 37°C in 50 mM Tris buffer (pH 7.5) containing 2 mM MgCl2. After incubation, the specimens were analyzed together with control holo-FldA by native PAGE followed by CBB staining.

As described above, some enzymatic activity was required for the conversion from holo-FldA to apo-FldA. To identify one of the activities involved in the conversion from holo-FldA to apo-FldA, we conducted experiments using cell lysates obtained from organisms cultured for 2 days (10^8.0 CFU/ml) and prepared with 50 mM Tris buffer (pH 7.5 or 8.6). In brief, cell lysates of pH 7.5 and 8.6 were applied to a Q-Sepharose (Sigma-Aldrich Co.) anion-exchange column and an SP Sepharose (Amersham Biosciences AB, Uppsala, Sweden) cation-exchange column, respectively, for fractionation with various concentrations of NaCl. After dialysis against 50 mM Tris buffer (pH 7.5), each fraction obtained from anion- or cation-exchange chromatography was incubated with purified holo-FldA overnight at 37°C in 50 mM Tris buffer (pH 7.5). The activity involved in the conversion from holo-FldA to apo-FldA was confirmed by monitoring the generation of apo-FldA from holo-FldA in native PAGE after the incubation. A sodium dodecyl sulfate-PAGE analysis revealed a protein with a molecular mass of 28 kDa at significant levels in the fractions exhibiting the activity involved in the conversion from holo-FldA to apo-FldA, though the expression levels of the same protein with a molecular mass of 28 kDa were lower than those of the activity involved in the conversion from holo-FldA to apo-FldA. PMF analysis (Shimadzu Co.) identified a 28-kDa protein (the activity involved in the conversion from holo-FldA to apo-FldA) with class C acid phosphatase from holo-FldA to apo-FldA via the phosphomonoesterase activity of HppA. (A) Holo-FldA (1 μg) was incubated in the presence or absence of HppA (0.05 μg) overnight at 37°C in 50 mM Tris buffer (pH 7.5) containing 2 mM MgCl2. After incubation, the specimens were analyzed together with control holo-FldA by native PAGE followed by CBB staining. The activity involved in the conversion from holo-FldA to apo-FldA was successfully obtained as a single protein with a molecular mass of 28 kDa from the fraction eluted with 0.2 M NaCl. PMF analysis (Shimadzu Co.) identified a 28-kDa protein (the activity involved in the conversion from holo-FldA to apo-FldA) with class C acid phosphatase (HppA) matching the HppA protein (calculated pI value, 9.41) of the *H. pylori* CZD 115 strain, with a top score of 111 in the Mascot Search (Matrix Science Ltd.).

A confirmatory PMF analysis identified the activity involved in the conversion from holo-FldA to apo-FldA as HppA. We thus inferred that the HppA-induced dephosphorylation of FMN plays an important role in the conversion from holo- to apo-FldA. To investigate further, we examined the generation of riboflavin (Rib) from FMN in holo-FldA reacted with HppA. A MALDI-TOF MS analysis (Shimadzu Co.) was performed to detect the mass spectrum of Rib or FMN in holo-FldA solution incubated in the presence or absence of HppA. The holo-FldA solution incubated without HppA exhibited the mass spectrum of FMN (identified with the mass spectrum of...
an authentic FMN), whereas the holo-FldA solution incubated in the presence of HppA exhibited the mass spectrum of Rib (identified with the mass spectrum of an authentic Rib) (Fig. 3A). This confirmed that the FMN within the FldA protein was dephosphorylated via the action of HppA, which in turn resulted in Rib. We next examined whether the phosphomonoesterase activity of HppA induced FMN dephosphorylation. As shown in Fig. 3B, holo-FldA was converted to apo-FldA in the presence of HppA without 4-methylumbelliferyl phosphate (MUP), a substrate for the phosphomonoesterase activity of a nonspecific acid phosphatase, whereas the HppA-induced conversion of holo-FldA to apo-FldA was inhibited in the presence of excessive MUP. These results confirm that the FMN in holo-FldA is hydrolyzed by the phosphomonoesterase activity of HppA and that the resulting Rib is released from the FldA protein.

HppA is a class C acid phosphatase characterized by four invariant aspartate residues (DDDD motif) within the most-conserved domain of the molecule (10). In this study, we attempted to purify HppA by cation-exchange chromatography of a culture supernatant obtained from *H. pylori*. It was not surprising to find HppA within the supernatant, given that the HppA protein of *H. pylori* has been detected in both the outer membrane fraction and whole-cell extracts (3). HppA has recently been characterized as a 5′/H11032 nucleosidase in *H. pylori* (8). The enzyme was also found to catalyze the hydrolysis of free FMN, albeit with a substrate specificity remarkably lower than its substrate specificity to 5′ nucleotides such as AMP (8). The above study had a limitation, however, as it did not examine the hydrolysis of FMN contained within the FldA protein via the phosphomonoesterase activity of HppA. Further investigation is necessary to determine the pH optimum and Michaelis constant for the enzyme activity of HppA in the FMN residing as a substrate within the FldA protein.

This work was supported in part by the Jichi Medical University Young Investigator Award.

REFERENCES