Isolation of Flavohemoglobin from the Actinomycete *Streptomyces antibioticus* Grown without External Nitric Oxide Stress

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A flavocytochrome protein was isolated from the actinomycete *Streptomyces antibioticus*. The purified protein contained protoheme and FAD, and its Mr was estimated to be 52,000. The absorption spectra in its resting oxidized, dithionite-reduced, carbon monoxide-bound, and oxygenated (O₂-bound) forms were characteristic of those of flavohemoglobin (Fhb). The N-terminal amino acid sequence showed high identities to those of other Fhb's. Furthermore, the actinomycete flavocytochrome scavenged nitric oxide in the presence of NADH. These results demonstrated that the flavocytochrome is the first Fhb purified from actinomycetes. The actinomycete Fhb was produced in *S. antibioticus* cells in large amounts without any external nitric oxide (NO) stress, which is indicative of a physiological function of Fhb other than detoxification of NO.

Key words: flavohemoglobin; actinomycetes; nitric oxide stress; *Streptomyces antibioticus*

Nitric oxide (NO) is a free radical and a molecule dangerous to organisms. Thus NO was previously known to occur biologically only in bacterial denitrification.¹) Recently, however, NO has been recognized to exhibit multiple and diverse biological functions, in mammals in particular. Nitric oxide synthase (NOS) from arginine, and controls blood pressure and is a messenger in the central and peripheral nervous systems. NO is also generated by the immune system to inhibit key enzymes in bacterial and other foreign cells, which include terminal oxidases, other dioxygen (O₂)-binding heme enzymes, and aconitase that contains an iron-sulfur center with a non-redox function.³)

In contrast to expanding understanding of the occurrence and physiological functions of NO in mammals, little is known of its occurrence and functions in microorganisms, although recent results of genome analyses have revealed the presence of NOS gene homologs in many bacteria.⁴–⁶) As for NO metabolism by microorganisms, only mechanisms for NO detoxification have been studied extensively.⁷,⁸) Nitric oxide reductase (Nor) reduces NO to nitrous oxide (N₂O) and is known to be involved in denitrification.¹) Bacterial Nor is associated with the respiratory chain and thus its primary physiological function is respiratory and not for detoxification. On the other hand, fungal Nor (P450nor) receives electrons directly from NAD(P)H⁹) and thus is not associated with the respiratory chain, although it is involved obligatorily in fungal denitrification.¹⁰) It is therefore evident that P450nor is not a respiratory but a detoxifying enzyme that scavenges NO that is formed as the result of nitrate respiration in fungal mitochondria.¹¹) In bacteria, flavohemoglobin (Fhb)⁷,⁸) and flavorubredoxin¹²) have recently been proposed as detoxifying enzymes for NO that are expressed in *Escherichia coli* cells in response to nitrosative (NO) stress. Fhb has been shown to exhibit NO dioxygenase activity with a reasonable catalytic turnover to convert NO to nitrate.¹³,¹⁴) But the stress is created artificially by NO-evolving agents or by adding NO itself. Therefore it is still obscure whether the stress against the enterobacterium occurs under the natural environment or not.

Fhb is widely found in microorganisms, bacteria,⁷,¹⁵,¹⁶) yeast,¹⁷) and fungi.¹⁸) Detoxification of NO is a potential physiological function of Fhb, as noted above. Some interaction of Fhb with bacterial denitrification is suggested although its real function in denitrification is not known.¹⁹) Actinomycetes have long been thought of as aerobic organisms and thus the occurrence of dissimilatory metabolism of nitrogen (nitrate) in actinomycetes was previously unknown. We found recently that some actinomycetes exhibit denitrifying activity.²⁰) The occurrence of dissimilatory nitrogen metabolism in actinomycetes was further supported by genome analysis, which revealed the presence of genes homologous to the dissimilatory nitrate reductase gene in *Streptomyces coelicolor*.²¹) Fhb
gene homologs are also found in the genomes of *S. coelicolor* and *Streptomyces avermitilis*, suggesting a general occurrence of dissipilatory nitrogen metabolism among actinomycetes. In this study we isolated Fhb protein from the denitrifying actinomycete *Streptomyces antibioticus*.

**Materials and Methods**

*Strain and media.* *Streptomyces antibioticus* B-546 was used throughout this study. Preculture was grown in a 50 ml test tube containing 10 ml of a meat extract medium (30 ml of glycerol, 10 g of meat extract (Kyokuto), and 5 g of yeast extract (Difco) in 1000 ml of distilled water, pH 7.2) and sealed with a cotton plug. After washing, the column was equilibrated with 50 mM sodium-potassium phosphate buffer (pH 7.0) containing 150 mM NaCl. The Relative molecular mass (*M*) of Fhb was determined by both SDS-polyacrylamide gel electrophoresis (PAGE) and gel-filtration. Gel-filtration was performed under non-denaturing conditions with FPLC equipped with a Superdex 200 column (Pharmacia, Sweden) equilibrated with 50 mM sodium-potassium phosphate buffer (pH 7.0) containing 150 mM NaCl. Molecular marker proteins were obtained from BIORAD (for SDS-PAGE; USA), Sigma (USA), and Wako (Osaka, Japan) (for gel-filtration).

**Table 1.** Purification of Fhb from *S. antibioticus*

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein (mg)</th>
<th>Total Fhb (nmol)</th>
<th>Specific content (nmol/mg)</th>
<th>Fhb recovery (%)</th>
<th>Purification (-fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soluble fraction</td>
<td>1981</td>
<td>1750</td>
<td>0.88</td>
<td>100</td>
<td>1.0</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>451</td>
<td>780</td>
<td>1.7</td>
<td>44.5</td>
<td>2.0</td>
</tr>
<tr>
<td>1st DEAE-Toyopearl</td>
<td>108</td>
<td>261</td>
<td>2.4</td>
<td>15.0</td>
<td>2.7</td>
</tr>
<tr>
<td>Phenyl-Sepharose</td>
<td>13.4</td>
<td>81</td>
<td>6.1</td>
<td>4.6</td>
<td>6.9</td>
</tr>
<tr>
<td>2nd DEAE-Toyopearl</td>
<td>2.1</td>
<td>39</td>
<td>13.8</td>
<td>1.6</td>
<td>15.7</td>
</tr>
</tbody>
</table>
The N-terminal amino acid sequence of the actinomycete Fhb was determined as follows. The purified, colored fraction (50 μg protein) was subjected to SDS-PAGE, and proteins in the gel after PAGE was transferred to a PVDF membrane (ATTO, Tokyo Japan) with a blotting instrument (Horizelot AE-6670P/N, ATTO). The membrane was then stained with Coumassie brilliant blue and the band with Mr of 52,000 was cut off and applied to an Applied Biosystem 492 Protein sequencer to determine its N-terminal amino acid sequence.

Protein was determined with a protein assay reagent (Bio-Rad Laboratories Inc., California, USA).

NO dioxygenase activity. It was shown that E. coli Fhb exhibits NO dioxygenase activity, which oxidizes NO to nitrate by combining NO and O2 with the supply of one electron derived from NADH. This activity was monitored by detecting NO concentrations in the reaction mixture with an ISO-NO Clark-type NO electrode (World Precision Instrument, USA). The reaction mixture contained in 20 mM potassium phosphate buffer (containing 1 mM DTT and 0.1 mM EDTA, pH 7.3) 0.1 mM NADH, 10 μM 3-(2-hydroxy-1-methyl-3-nitrosohydrazino)-N-methyl-1-propanamine (NOC 7, with a half-lifetime of 5 min; Dojin, Kyoto Japan), and 0.36 μM Fhb.

Results and Discussion

Mr determination and heme-staining
The purified, colored protein was subjected to SDS-PAGE and gel-filtration (non-denaturing conditions) (Fig. 1). The Mr value estimated by both methods agreed well at 52,000, suggesting that the colored protein has a monomer structure.

Cofactors and absorption spectra
The purified protein exhibited an absorption spectrum with peaks at 407, 540, and 631 nm in its resting form, which is characteristic of a hemeprotein (Fig. 2A). The hemeprotein also exhibited fluorescence with a peak around 530 nm (excited at 450 nm), which is characteristic of flavin (data not shown). Further, a weak absorption band was observed around 448 nm (Fig. 2A) in its resting form, which is probably derived from oxidized flavin. Upon reduction with dithionite, the resting spectrum was replaced with new ones with peaks at 426 and 556 nm, and the band around 448 nm disappeared. These results are consistent with reduction of the heme and flavin in the protein. The hemeprotein was able to bind carbon monoxide (CO), which was confirmed by the spectrum obtained after dithionite-reduction and exposure to CO, exhibiting its α-, β-, and γ-(Soret) bands at 563, 538, and 420 nm respectively. The bound heme was identified as protoheme by the pyridine ferrohemochrome method, which gave a characteristic spectrum with peaks at 419, 525, and 557 nm (Fig. 2B). The heme content of the purified protein was estimated to be 0.82 mol per mol of protein by the spectrum. The hemeprotein was also shown to contain 1.28 mol of FAD per mol of protein by its fluorescence spectra. It would thus appear that the protein contains 1 mol each of protoheme and FAD per mol of protein. The rather lower content of heme probably depends on the property of the flavocytochrome to release heme easily.

The flavocytochrome was shown to be capable of reduced with NADH, as was confirmed by the spectral change in the cytochrome attained when incubated with NADH in the presence of oxygen (O2) to give new absorption peaks at 414, 543, and 575 nm. This spectrum is characteristic of the oxygenated form of hemoglobin.

Fig. 1. Mr Determination of Actinomycete Fhb.

The purified color fraction was subjected to SDS-PAGE (A) or gel-filtration (B). Standard proteins were myosin (Mr, 218,800), β-galactosidase (135,200), bovine serum albumin (85,700), carbonic anhydrase (40,900), and soybean trypsin (32,000) in A; and bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), and trypsin inhibitor (20,000) in B.
the O₂-bound ferrous form). These results for the cofactors and spectral properties of the purified, colored protein of *S. antibioticus* support that the protein is a member of Fhb.

**N-terminal amino acid sequence**

The amino (N)-terminal amino acid sequence of the actinomycete Fhb was determined and aligned with those of other members of Fhb (Fig. 3). The sequence of *S. antibioticus* Fhb showed identities to various extents with those of other members of the Fhb family. A sequence region highly conserved among these forms of Fhb was observed between the 10th and 15th amino acid residues. *S. antibioticus* Fhb showed higher identity or similarity as against Fhbs of other actinomycete strains (*S. coelicolor* and *S. avermitilis*), with the exception of one isozyme of *S. avermitilis*. It is interesting that this isozyme (Save60) is different from other Fhbs in N-terminal amino acid sequence and molecular weight (above 60,000, in contrast to other Fhbs, most of whose...
Mr distribute between 40,000–45,000, implying a potential physiological function different from those of other Fhbs.

**NO-scavenging activity of S. antibioticus Fhb**

The NO-donating agent NOC 7 was shown to evolve NO in the reaction vessel as monitored with an NO electrode (Fig. 4). When the agent was incubated with both S. antibioticus Fhb and NADH, NO did not evolve, or disappeared rapidly just after Fhb was added, as shown in Fig. 4. The results show that S. antibioticus Fhb exhibits an NO scavenging activity. We could not detect, however, nitrate, the expected product of NO dioxygenase. Determination of NO dioxygenase activity would be rather difficult since the two substrates, NO and dioxygen (O₂), can also react chemically to form NO₂, whereas Fhb might exhibit Nor activity under anoxic conditions. Identification of the reaction product due to actinomycete Fhb is now underway.

**Estimation of Fhb in the crude cell extract (soluble fraction)**

Fhb in the soluble fraction as well as the purified protein was described in Materials and Methods. But the presence of CO-binding hemeproteins other than Fhb in the crude preparation cannot be ruled out. So we estimated Fhb and other hemeproteins in the crude extract of the S. antibioticus cells. Heme staining of the gel after a native PAGE of the fraction revealed a thick major band with the same mobility as Fhb (Fig. 5A). The absorption spectrum of the soluble fraction showed a major peak around 415 nm, as shown in Fig. 5B. Upon the bubbling of CO gas, the peak was replaced with a sharper peak around 420 nm, showing that these absorption bands (415- and 420 nm species) are derived from a CO-binding hemeprotein(s) (P420). Further addition of a reductant (dithionite or NADH) prior to bubbling CO did not increase, or decreased, the concentration of the P420 species. This means that the heme in the soluble fraction is in a reduced (ferrous) form and that the 415 nm species is probably derived from the oxygenated (O₂-bound ferrous) Fhb. A non-autoxidizable nature is a highly characteristic feature of hemoglobin. The decrease in the P420 content by dithionite treatment probably arose from a partial denaturation of the Fhb protein. These results clearly demonstrated that almost all of the CO-binding pigment (P420) in the soluble fraction is Fhb, and that other P420 species, if present, are negligible.

**Conditions for induction of Fhb**

Most papers on Fhb associate the physiological function of Fhb with NO stress, that is, detoxification of the dangerous molecule NO, whereas a few papers suggest other roles. In the case of the yeast Saccharomyces cerevisiae, it has been suggested that Fhb functions in coping with the oxidative stress. In Alcaligenes eutrophus and the fungus Fusarium oxysporum, it has been suggested that Fhb interacts with denitrification, which is also supported by a higher sequence identity between the bacterial and fungal Fhbs in the N-terminal sequence alignment (Fig. 3). We previously showed that S. antibioticus exhibits denitrification activity. Therefore, we examined the effects of NO stress or denitrification on the production of Fhb in S. antibioticus cells.

As shown in Table 2, the Fhb content of the soluble fraction was not affected by the addition of nitrite or the NO-donating reagent (NOC 18). The Fhb content in each fraction exceeded 5% of total soluble proteins. It is thus concluded that Fhb is produced in large amounts in S. antibioticus cells, and this is apparently independent of NO stress or denitrifying conditions.
This paper is the first to report purification of Fhb protein from actinomycetes and to demonstrate the NO-scavenging activity of actinomycete Fhb. The conditions for production of Fhb in *S. antibioticus* are also unique in that they are independent of external NO stress or denitrification. The amount of Fhb produced was more than 5% of the soluble proteins. These results are indicative of some important physiological function of actinomycete Fhb in cell growth.

Acknowledgments

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References


**Table 2.** Production of Fhb in *S. antibioticus*

<table>
<thead>
<tr>
<th>Additive</th>
<th>Specific content of Fhb (µmol/mg protein)</th>
<th>Percent against total proteins (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td>1.12</td>
<td>5.8</td>
</tr>
<tr>
<td>nitrite (5 mM)</td>
<td>1.33</td>
<td>6.9</td>
</tr>
<tr>
<td>NOC18 (1 mM)</td>
<td>0.98</td>
<td>5.1</td>
</tr>
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</table>


