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Identification of a Blue Copper Protein from *Hyphomicrobium denitrificans* and its Functions in the Periplasm

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It has been known that the methylotrophic denitrifying bacteria have the specific electron transfer chains, involving in 'methanol oxidation' and 'denitrification', in the periplasm. Recently, a unique blue copper protein (HdBCP) has been isolated from the methanol-grown methylotrophic denitrifying bacterium, *Hyphomicrobium denitrificans*. HdBCP is a 14.5 kDa protein and contains one copper atom in the molecule. The electronic absorption spectrum of HdBCP exhibits two absorption maxima near 450 and 750 nm comparable with the intense 600 nm band ($\varepsilon_{450}/\varepsilon_{600}$ = ca. 0.9). The rhombic electron paramagnetic resonance spectrum shows clearly that the copper centre is a 'perturbed' type 1 copper geometry. Stopped-flow kinetics indicates that HdBCP accepts efficiently an electron from cytochrome c_L ($k_2 = 4.0 \times 10^6\,M^{-1}\,s^{-1}$ at 25.0°C), which is a physiological electron acceptor for methanol dehydrogenase. According to cloning and DNA sequencing of the structural gene, the deduced amino acid sequence shows significant similarities with pseudoazurins, which are a physiological electron donor for Cu-containing nitrite reductase from the denitrifying bacteria. Based on these results, we discuss the role of HdBCP in the electron-flow system, which link 'methanol oxidation' and 'denitrification' together.

Key words: blue copper protein, denitrification, electron transfer, methylotroph, type 1 copper.

Abbreviations: BCP, blue copper protein; NIR, nitrite reductase; HdNIR, Cu-containing nitrite reductase from *Hyphomicrobium denitrificans*; MDH, methanol dehydrogenase; Cyt c_L. cytochrome c_L; Cyt c_{550}, cytochrome c_{550}; Am, amicyanin; Az, azurin; PAz, pseudoazurin; Hd, *Hyphomicrobium denitrificans*; Af, *Alcaligenes faecalis*; Ac, *Achromobactor cycloclastes*; Ax, *Alcaligenes xylosoxidans*; Pp, *Paracoccus pantotrophus*; Me, *Methylobacterium extorquens* AM1; EPR, electron paramagnetic resonance; ET, electron transfer.

Hyphomicrobium denitrificans is a methylotrophic denitrifying bacterium, capable of growth with methanol as a sole carbon source (*1*, *2*). Methanol is oxidized to formaldehyde by the reduction of pyrroloquinoline quinone (PQQ) in methanol dehydrogenase (MDH), and then the reduced PQQ transfers electrons to a physiological electron acceptor cytochrome c_L (Cyt c_L) (*3–8*). Cyt c_L is subsequently oxidized by cytochrome c_{550} [Cyt c_{550} corresponds to cytochrome c_H from methylotrophic bacteria (*7*, *9–11*)]. Moreover, copper-containing nitrite reductase (NIR) and nitrous oxide reductase (N_2OR) involving in denitrification have been also purified from Hd cells grown on methanol (*12–14*). Recently, X-ray crystal structure of novel hexameric HdNIR has been determined (*15*). The molecular structure reveals a trigonal-prism-shaped homohexamer (a tightly associated dimer of trimers), in which the monomer consisting of 447 residues and three Cu atoms are organized. NIR is a key enzyme in denitrification, catalyzing the first step that leads to gaseous products ($NO_2^- + e^- + 2H^+ \rightarrow NO + H_2O$). The efficient electron donation from the specific electron donor protein to the enzyme is necessary for the catalytic cycle. In the case of Hd, the electron donor protein is Cyt c_{550} (*13*, *15*), which is a physiological electron acceptor for Cyt c_L. Therefore, the electron derived from methanol flows to HdNIR constituting the nitrate respiration system in the periplasmic space of a methylotrophic denitrifying bacterium, Hd as follows:

$$MeOH \rightarrow MDH \rightarrow Cyt\ c_L \rightarrow Cyt\ c_{550} \rightarrow HdNIR \rightarrow NO_2^-$$

In general, three bacterial blue copper proteins (BCPs), amicyanins (Am) (*16–23*), azurins (Az) (*24–29*) and pseudoazurins (PAz) (*16*, *17*, *30*, *31*), have been found in several methylotrophic or denitrifying bacteria. These are small electron transfer (ET) proteins characterized by an intense electronic absorption band near 600 nm (blue band) and a small hyperfine coupling constant (5–7 mT) in EPR spectra, which originate in the type 1 copper. The visible absorption spectra of Az and Am reveal a weak 450 nm band compared with the blue band ($\varepsilon_{450}/\varepsilon_{blue} = 0.1$–0.2) (*16*, *18*, *20*, *26*), while that of PAz exhibits two clear absorption maxima near 450 and 750 nm, compared with the most intense blue band ($\varepsilon_{450}/\varepsilon_{blue} = 0.4$–0.5) (*16*, *31*). The EPR signals of Az and Am show axial symmetries, but that of PAz indicates a rhombic symmetry.

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Blue Am is located in the periplasm of methylotrophic bacteria such as *Paracoccus denitrificans* (*20*, *23*) and *Methylobacterium extorquens* AM1 (Me) (*16–18*), being the smallest BCP having 99–105 amino acid residues. Its principal function is to accept electrons from methylamine dehydrogenase (MADH, EC 1.4.99.3) containing tryptophan tryptophylquinone (TTQ) and to transfer them to one or more cytochromes. On the other hand, two Az's (Az-iso1 and Az-iso2) have been identified from the obligate methylotroph *Methylomonas* sp. strain J isolated from garden soil by selection on a medium containing methylamine as the sole carbon source (*24*, *25*). Both Az's are expressed when the cells are grown on methylamine, but only Az-iso1 is detected when methanol is used instead of methylamine. Recently, it has been demonstrated that Az-iso2 is a direct electron acceptor for MADH *in vivo* (*32*).

In denitrification, greenish-blue PAz is obviously an electron donor for green Cu-containing NIRs (*33–36*). The former is a basic protein, and the latter is acidic. The second-order rate constant (k_{ET}) of intermolecular ET from *Achromobacter cycloclastes* (Ac) PAz to its cognate NIR was estimated to be $7.3 \times 10^5\,M^{-1}\,s^{-1}$ (pH 7.0) by cyclic voltammetry (*33*). The electrostatic interaction between the basic amino acid residues of the PAz from *Alcaligenes faecalis* (Af) S-6 and the acidic amino acid residues of its cognate NIR is proposed by X-ray crystal structure analyses and kinetics (*35*). Moreover, blue Az is also expressed in some denitrifying bacteria under anaerobic growth. It has been suggested that both Az and cytochrome c_{551} (Cyt c_{551}) receive electrons from bc_1 complex and donate them to NIR (*37*). Az was also reported as an electron donor for the Cu-containing NIRs from *Pseudomonas aureofaciens* (*38*) and *Alcaligenes xylosoxidans* (Ax) NCIMB 11015 (*27*). However, the cyclic voltammetries of the Az-I and Az-II from Ax exhibited little response in the presence of the enzyme and nitrite, indicating the very slow ET processes ($k_{ET} < 10^4\,M^{-1}\,s^{-1}$) (*36*). An *in vivo* approach with *P. aeruginosa* mutants deficient in one or both Cyt c_{551} and Az have shown that an electron donor for heme cd_1-containing NIR is Cyt c_{551}, and not Az (*39*).

Recently, we have found a unique BCP in the periplasmic fraction of Hd cells grown on methanol. The protein was rapidly reduced with a physiological electron acceptor for MDH, Cyt c_L, under the physiological conditions. The spectroscopic characterization and the stopped-flow kinetic measurements of the ET between the protein and Cyt c_L were performed. Furthermore, the structure gene was cloned and the deduced amino acid sequence was analysed.

MATERIALS AND METHODS

Preparation of the Blue Copper Protein from H. denitrificans—For isolation of Hd blue copper protein (HdBCP), Hd A3151 was cultured as previously described (*1*). The cells suspended in 40 mM Tris-HCl buffer (pH 7.5) containing 0.5 mM phenylmethanesulfonyl fluoride were sonicated at 180 W for 30 min. The resulting cell debris was removed by centrifugation at 15,000 rpm for 1 h at 4°C. After centrifugation, the supernatant was applied directly onto a Super Q-Toyopearl column (5 × 15 cm, Tosoh) pre-equilibrated with 40 mM Tris-HCl buffer (pH 7.5). The column was washed with the four volumes of the same buffer, and HdBCP was not adsorbed to the column. The flow-through fraction was further applied onto a Q-Sepharose Fast Flow column (2.5 × 25 cm, Amersham). HdBCP was passed through the column again. Ammonium sulphate was added to a final concentration of 60% saturation to the resulting flow-through fraction (approximately. 1 l). The resulting precipitate was removed by centrifugation (8,000 rpm for 40 min at 4°C). The supernatant was applied onto a Phenyl-Sepharose Fast Flow column (2.5 × 25 cm, Amersham) pre-equilibrated with 40 mM Tris-HCl (pH 7.5) containing a 60% saturated concentration of ammonium sulphate. HdBCP adsorbed on the column was eluted with a linear gradient from 60 to 0% saturated concentration of ammonium sulphate in the same buffer. The HdBCP-containing fractions were collected and then dialyzed with 10 mM potassium phosphate (pH 6.0) for 12 h. The dialyzed sample was applied onto a CM-Sepharose Fast Flow column (2.5 × 25 cm, Amersham) pre-equilibrated with 10 mM potassium phosphate buffer (pH 6.0). HdBCP was eluted with a linear gradient from 10 to 200 mM potassium phosphate (pH 6.0). The collected HdBCP fractions were dialyzed with 10 mM potassium phosphate buffer (pH 6.0) and then applied onto a Resource S column (1.6 × 3.0 cm, Amersham). HdBCP was eluted with a linear gradient from 10 to 80 mM potassium phosphate buffer (pH 6.0). The fractions were collected, concentrated and desalted with a Centriprep-YM10 (Millipore). The purity was estimated to be 90% over by SDS-PAGE. The yield was ca. 1.0 mg/60 g of wet cells.

Physical Measurements—The electronic absorption and CD spectra were measured at 25°C with a Shimadzu UV-2450 spectrophotometer and a J-500A spectropolarimeter (JASCO), respectively. The EPR spectrum was recorded with a JEOL JES-FE1X X-band spectrometer at 77 K. The copper content was determined with a Nippon Jarrel Ash AA-880 Mark-II atomic absorption spectrophotometer. Cyclic voltammetric analysis was carried out using a Bio analytical Systems Model CV-50W voltammetric analyser with a three-electrode system consisting of a Ag/AgCl reference electrode, a gold wire counter electrode, and a bis(4-pyridyl)disulfide-modified gold working electrode under Ar atmosphere.

Stopped-Flow Kinetics—Stopped-flow experiments were carried out at 25.0°C with a RA-2000 stopped-flow spectrophotometer (Otsuka Electronics). The rapid oxidation of reduced Cyt c_L with HdBCP was monitored at 417 nm, which is the wavelength of the maximum peak ($\Delta\varepsilon = 57.2\,mM^{-1}\,cm^{-1}$) in the difference absorption spectrum between the ferrous and ferric forms of Cyt c_L. The extinction coefficient of HdBCP is $1.7\,mM^{-1}\,cm^{-1}$ at 417 nm. To determine the kinetic constants, the concentrations of HdBCP were up to 10-fold greater than that of Cyt c_L. Pseudo-first-order rate constants were calculated by non-linear regression with a IgorPro version 5.0 (WaveMetrics). The data obtained were the average of at least three experiments (the errors were normally within 5%).

N-terminal Amino Acid Sequencing of Mature HdBCP and Five Tryptic-Digested Peptides—The purified HdBCP (200 μg) was digested with TPCK-treated trypsin (2 μg) at 30°C for 3 h in 100 mM Tris-HCl (pH 8.0) containing 2 M Urea. The five tryptic-digested peptides were separated with a YMC-Pack ODS-A302 reverse-phase column using a 0.1% (v/v) trifluoroacetic acid solution and a 95% acetonitrile solution containing 0.1% trifluoroacetic acid. HPLC was carried out at a flow rate of 0.25 ml/min by continuous monitoring of the 215 nm absorbance. The N-terminal amino acids of the purified mature HdBCP and the five peptide fragments were determined using a Model 477A protein sequencer (ABI).

Cloning of the Structural Gene of HdBCP—The N-terminal amino acid sequence of mature HdBCP was A-E-H-I-V-E-M-R-N-K-D-D-A-G-N-T-M-V-F-Q showing a significant similarity (ca. 50%) with that of *P. pantotrophus* PAz. One of the five peptides was K-R-L-D-G-E-I-A, showing a significant similarity with the C-terminal sequence of PAz. PCR with the Hd genomic DNA as a template was preformed using two primers (5′-cacatagtg gagatgcgcaacaaggacgac-3′ and 5′-ggcgatctcgccgtc cagccgctt-3′) designed manually from both N- and C-terminal amino acid sequences and from DNA homology analysis in all PAz's nucleotide sequences. The resulting ca. 360-bp DNA fragment was amplified. The fragment was inserted into the pTA2 vector (Toyobo). The resulting plasmid, pTABCP was digested with *Eco*RI. The ca. 360-bp DNA fragment was inserted into the *Eco*RI site of plasmid pUC119. The full length of the insert DNA was sequenced. Since the sequence analysis clearly showed that the insert DNA is a partial structural gene for HdBCP, the fragment DNA was used as a specific probe for the next colony hybridization step. The *Eco*RI-digested Hd genome DNA fragments (size fraction 2 to 4 kbp) were ligated with Charomid 9-36 DNA (Nippon Gene) linearized with *Eco*RI. The mixture was used directly for *in vitro* packaging using *In vitro* Packaging Kit LAMBDA INN (Nippon Gene) with *Escherichia coli* DH5a as a host. Colony hybridization was carried out by use of AlkPhos Direct labelling Hybridization kit (GE Healthcare) for the total 2,000 colonies obtained.

RESULTS AND DISCUSSION

Purification of H. denitrificans Blue Copper Protein (HdBCP)—The two cytochromes *c* (Cyt c_L and Cyt c_{550}) are contained in methanol-grown Hd A3151 (*8*). At the final purification step, both HdBCP and Cyt c_{550} are adsorbed to a cation-exchange Resource S column at pH 6.0 (10 mM potassium phosphate buffer), and then HdBCP is eluted following weak basic Cyt c_{550} with a linear gradient from 10 to 80 mM potassium phosphate buffer (pH 6.0) (Fig. 1). The yield of HdBCP was ca. 1.0 mg/60 g of wet cell even if in a methylamine-containing medium (instead of methanol). Interestingly, the yield was dramatically increased, when the ratio of Cu to Fe in the medium was increased (e.g. Cu, 130 μM and Fe, 0.35 μM). The yield of HdBCP from the cells grown on the modified medium composition was about 30 mg/60 g wet cell. The purified HdBCP gives a single band at a molecular mass of 14.5 kDa on the SDS-PAGE (Fig. 1, inset). The copper content was determined to be 0.95/mol of the protein by an atomic absorption spectroscopy.

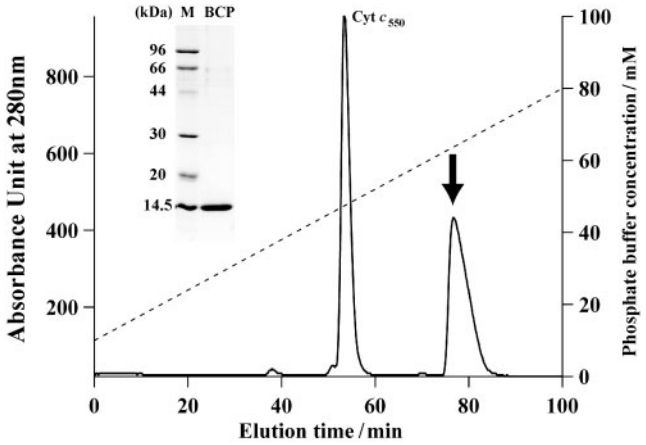

Fig. 1. **The Resource S column chromatograph for the purification of HdBCP.** Allow indicates the elution band of HdBCP. The broken line means a linear gradient from 10 to 80 mM potassium phosphate buffer (pH 6.0). Inset, SDS-PAGE of purified HdBCP (2 mg). M is a molecular weight marker set.

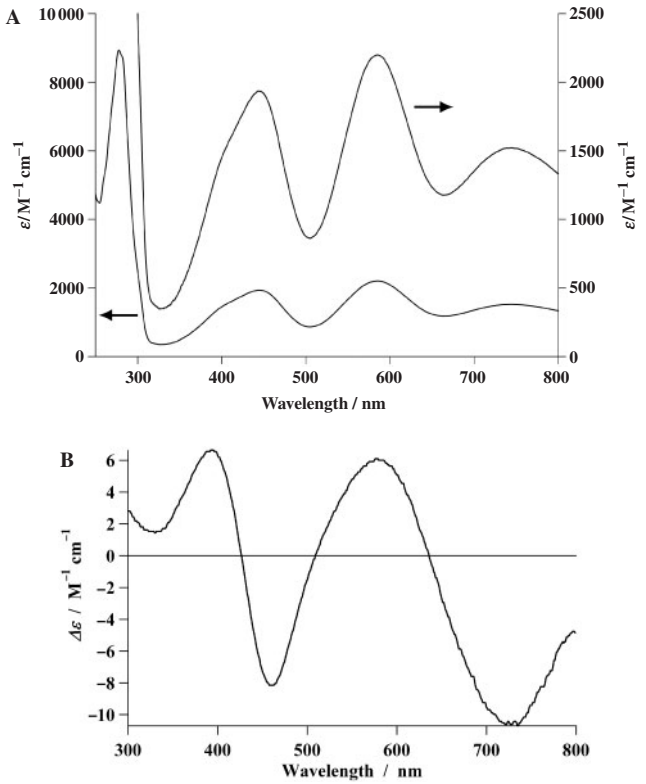

Fig. 2. **Electronic absorption (A) and CD (B) spectra of HdBCP in 10 mM potassium phosphate buffer (pH 6.0) at room temperature.**

Spectroscopic Characterization of HdBCP—Figure 2 shows the electronic absorption (A) and CD (B) spectra of HdBCP in 10 mM phosphate buffer at pH 6.0.

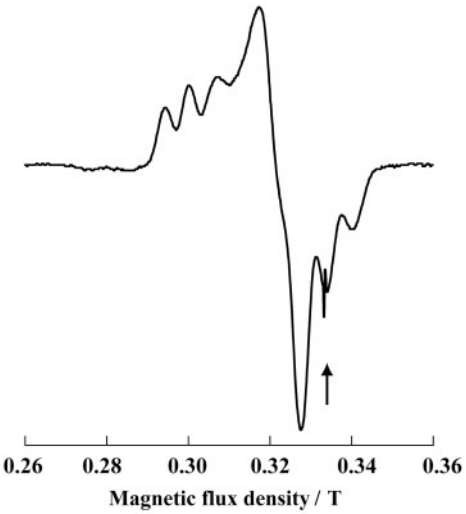

Fig. 3. **EPR spectrum of HdBCP in 10 mM potassium phosphate buffer (pH 6.0) at 77 K. Allow indicates an internal standard signal (TCNQ).**

The visible absorption spectrum exhibits three peaks at 444 ($\varepsilon = 1{,}940$), 585 ($\varepsilon = 2{,}200$) and 745 nm ($\varepsilon = 1{,}520\,M^{-1}\,cm^{-1}$). The intense ca. 600 nm band and the spectral pattern of HdBCP are quite similar to those of cucumber basic protein (CBP) from plant (*40–42*) rather than those of the well-known BCPs (Am, Az and PAz) from the methylotrophic or denitrifying bacteria. The visible CD spectrum shows four extrema at 394 ($\Delta\varepsilon = 6.64$), 460 ($\Delta\varepsilon = -8.14$), 578 ($\Delta\varepsilon = 6.09$) and 728 nm ($\Delta\varepsilon = -10.6\,M^{-1}\,cm^{-1}$), and zero-crossings occur at 427, 509 and 636 nm. Therefore, the characteristic intense electronic absorption band around 450 nm is caused by intense positive and negative CD transitions at 394 and 460 nm, respectively. The ca. 450 nm electronic absorption band most likely consists of (His)Nδ1 $\rightarrow$ Cu(II), (Met)Sδ $\rightarrow$ Cu(II) and (Cys)Sγ (pseudo-σ) $\rightarrow$ Cu(II) charge-transfer transitions (*40*, *42*, *43*). The 77-K EPR spectrum of HdBCP in Fig. 3 exhibits a typical rhombic character ($g_z = 2.21$, $g_y = 2.08$, $g_x = 2.02$, $A_z = 6.0$ mT and $A_x = 6.8$ mT) and shows significant similarities to those of PAz [$g_z = 2.24$, $g_y = 2.09$, $g_x = 2.02$, $A_z = 5.5$ mT and $A_x = 6.8$ mT (*44*)] and CBP [$g_z = 2.22$, $g_y = 2.07$, $g_x = 2.02$, $A_z = 6.0$ mT and $A_x = 6.0$ mT (*40*)]. These spectral features are often observed in 'perturbed' BCPs, which exhibit substantially different spectral profiles from those of plastocyanin and Am having the 'classic' blue copper site (*42*). Therefore, HdBCP probably has a copper ligand set (Cys, 2His and Met) with shorter axial Cu–S (Met) and longer equatorial Cu–S (Cys) bonds than those of the 'classic' blue copper site, which is characterized as a 'perturbed' type 1 copper (*45–47*).

Intermolecular Electron Transfer Between HdBCP and Cyt c_L—The cyclic voltammogram of HdBCP shows a midpoint potential ($E_{1/2}$) of +277 mV versus NHE at pH 6.0. To observe the electron-accepting ability of HdBCP, the reduction of HdBCP was monitored spectroscopically in the presence of one or both MDH and Cyt c_L (Fig. 4A). At first the oxidized HdBCP was mixed with MDH. The visible spectrum was not so changed.

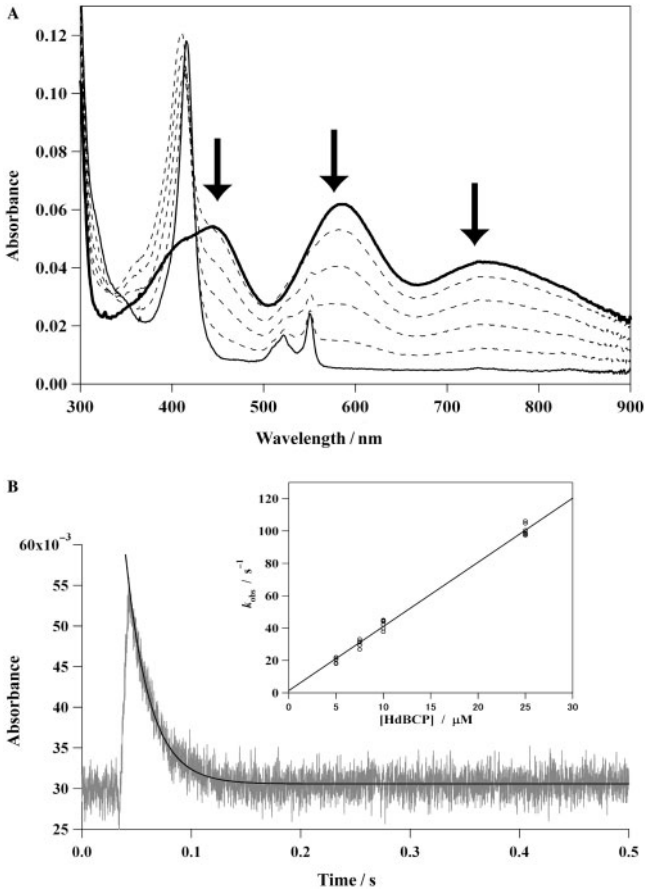

Fig. 4. **Intermolecular ET reaction between HdBCP and Cyt c_L.** (A) Bleaching of the visible absorption spectrum of HdBCP with Cyt c_L and MDH. Thick solid line: the visible absorption spectrum of the mixture of 25 μM HdBCP and 1 μM MDH in 10 mM potassium phosphate buffer (pH 6.0) containing 400 mM methanol. Broken line: the spectra with the 1 min interval, after 1 μM of Cyt c_L was added to the mixture. Thin solid line: after 5 min. (B) The rapid decay curve observed using stopped-flow spectrophotometer. Ten micromolar HdBCP and 0.5 μM Cyt c_L were contained in 10 mM potassium phosphate buffer (pH 6.0) containing 200 mM NaCl. Inset: The plot of k_{obs} versus HdBCP concentration.

However, further addition of Cyt c_L to the mixture results in rapid bleaching of the blue band of HdBCP. The findings clearly indicate that HdBCP cannot accept electrons directly from MDH without Cyt c_L. The detailed kinetics of intermolecular ET between HdBCP and Cyt c_L has been investigated by a stopped-flow absorption spectrophotometer (Fig. 4B). The k_{obs} values were obtained under the conditions that the reaction mixture contains 0.5 μM Cyt c_L and 5–25 μM HdBCP in the presence of 200 mM NaCl at pH 6.0. The rapid oxidation of Cyt c_L is monophasic and obeys pseudo-first-order kinetics. From the slope of the plot of k_{obs} versus HdBCP concentration, the second-order rate constant (k_2) was determined to be 4.0 (± 0.1) $\times 10^6\,M^{-1}\,s^{-1}$ at 25.0°C. Interestingly, the rate constant was similar to that of the intermolecular ET between Cyt c_L and Cyt c_{550} [6.0 (± 0.5) $\times 10^7\,M^{-1}\,s^{-1}$ at 25.0°C, manuscript in preparation; Nojiri, M. *et al*. (*8*)].

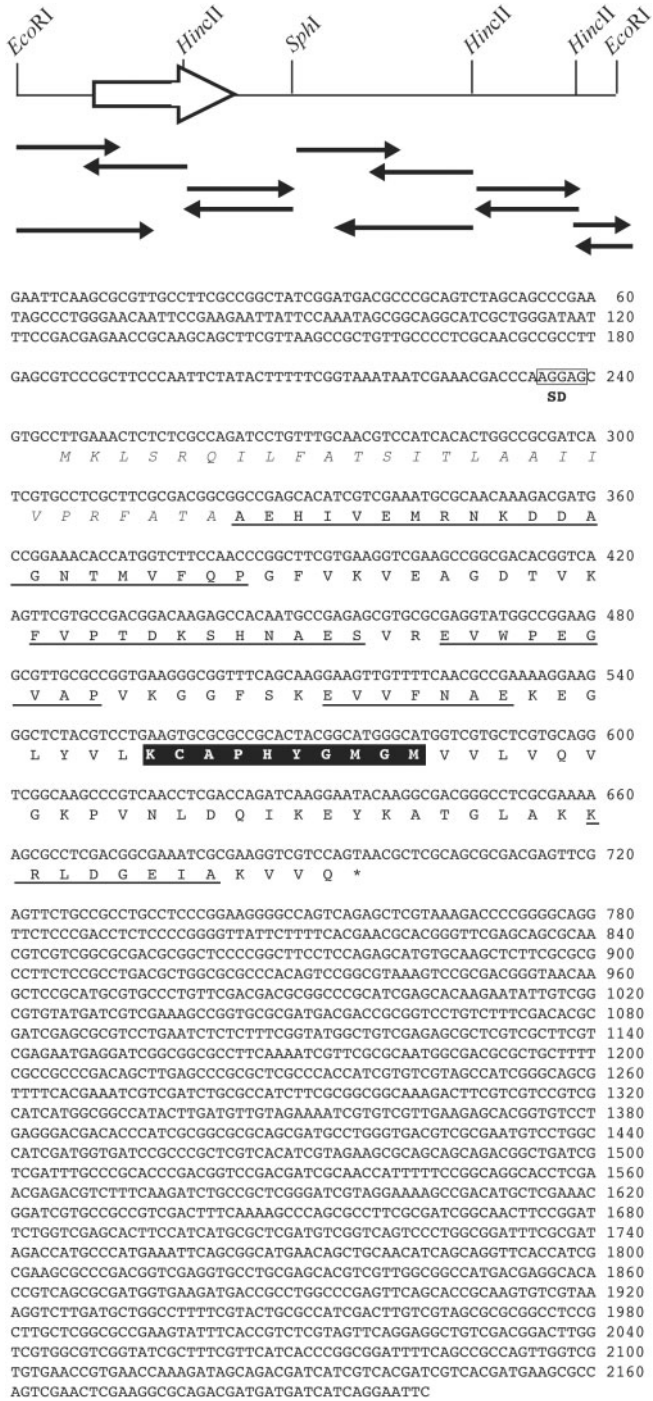

Fig. 5. **Nucleotide and deduced amino acid sequence of HdBCP.** Upper: schematic figure of the 2.2 kbp *Eco*RI fragment. Thick white allow represents the HdBCP coding region. Black allows represent the sequencing strategies. Lower: nucleotide and deduced amino acid sequence of HdBCP. The deduced amino acid sequence is shown below as a single-letter code. A potential ribosome-binding site (SD) is shown with a box. The analysed N-terminal amino acid sequences are shown with the thin underlines. The deduced signal peptide region is shown in italic. The specific motif [K]-[C]-[T/A]-[P]-[H]-x-[G/A/M]-[M]-[G/S]-[M] for PAz (*48*) is shown in white on a black background. Nucleotide sequence data reported are available in the DDBJ/EMBL/GenBank databases under accession number AB297894.

Cloning of the HdBCP Structural Gene and the Deduced Amino Acid Sequence—To obtain the amino acid sequence information on HdBCP, cloning of the structural gene was performed (MATERIAL AND METHODS). Using the PCR amplified ca. 360 bp DNA fragment as a specific probe, the HdBCP structural gene was screened from a partial genomic library of DNA fragments (ca. 2.0–4.0 kbp) obtained by digestion with *Eco*RI. One positive clone containing ca. 2.2 kbp insert DNA was obtained from colony hybridization and the full length of the fragment was sequenced. BLAST database searching shows obviously that there is one complete open reading frame (ORF) in the fragment (Fig. 5). The deduced amino acid sequence from the nucleotide sequence located at 323 bp downstream region of the *Eco*RI site completely corresponds with the N-terminal amino acid sequence of mature HdBCP, A-E-H-I-V-E-M-R-N-K-D-D-A-G-N-T-M-V-F-Q-. One rare initial TTG codon was found in the upstream region of the GCC codon of the N-terminal Ala. The ribosome-binding site (AGGAG) located at 6 bp upstream region of the TTG codon suggests that the ORF of the HdBCP structural gene includes 452 bp encoding 151 amino acid residues. Ala27 is the N-terminus of mature HdBCP (calculated Mr = 13,570) and is preceded by the signal peptide composed of 26 amino acid residues. All of the partial amino acid sequences determined were definitely found in the complete amino acid sequence deduced from the nucleotide sequence (Fig. 5, underlined). A common type 1 copper-binding motif [Cys79-$(X)_n$-His82-$(X)_n$-Met87 in the mature HdBCP sequence] is clearly found in the deduced amino acid sequence of mature HdBCP. The HdBCP sequence shows significant similarities with those of PAz's from various methylotrophic and denitrifying bacteria, *Paracoccus pantotrophus* (Pp) (50% identity), Af (41%), Ac (43%) and Me (45%). A characteristic region containing two short helices region at the C-termini of all PAz's, is also observed in the C-terminus of HdBCP (Lys95 to Gln124). Furthermore, according to the analysis using the newly designed protein signatures for the different BCPs (*48*), the specific motif [K]-[C]-[T/A]-[P]-[H]-x-[G/A/M]-[M]-[G/S]-[M] for PAz is completely conserved in the HdBCP sequence. From these results, we identified HdBCP with PAz. The amino acid sequence alignment of Hd PAz (HdPAz) is shown with those of Paz's from the denitrifying bacteria in Fig. 6. The possible copper ligands are His41, Cys79, His82 and Met87.

In conclusion, we have found and characterized a unique BCP from Hd. The spectroscopic character of HdBCP shows typical 'perturbed' type 1 copper features in the electronic absorption, CD and EPR spectra. The stopped-flow kinetics of ET between HdBCP and Cyt c_L indicates that not only Cyt c_{550} but also HdBCP function as an excellent electron acceptor for Cyt c_L in the periplasmic space. Moreover, the nitrite-reducing activity of HdNIR has been also assayed directly by using reduced HdBCP as an electron donor. The catalytic turnover (k_{cat}) is estimated to be 24 s^{-1} in 20 mM phosphate buffer (pH 6.5) containing 100 μM HdBCP. This result suggests that HdBCP also serves as an electron donor for NIR. This bacterium perhaps

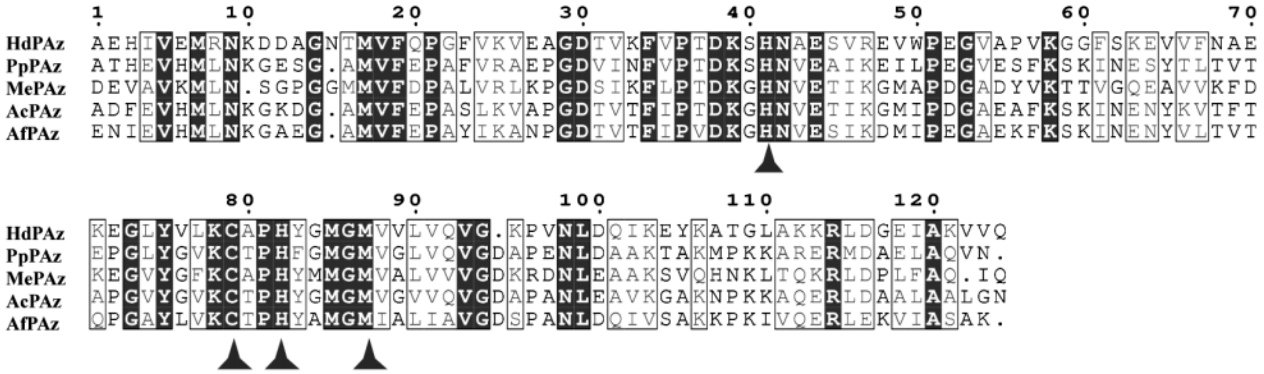

Fig. 6. **Alignment of the deduced amino acid sequences of HdPAz and four PAz's.** Their amino acid sequences were aligned using the ESPript online site (http://espript.ibcp.fr/ESPript/ESPript/). A similarity of the amino acid sequences of HdPAz with those of four PAz's was computed with the default parameter set (Similarity Groval Score, 0.7 and Similarity Matrix, Risler). Conserved residues are in the boxes, identical residues among five sequences are indicated by white letters on black backgroundand grey letters indicates similar residues. The Cu ligands are marked with black triangles.

maintains the important ET processes from 'methanol oxidation' initialized by MDH to the 'nitrate respiration (denitrification)' even under the conditions of environmental stress such as poor Fe environment. Similar observations by the genetic approach have been reported on a methylotrophic denitrifying bacterium, *P. denitrificans* (*49*). Cloning and sequence analysis clearly shows that HdBCP is a kind of PAz. In the present study, the stopped-flow kinetics of the ET process between PAz and Cyt c_L was first observed in the methylotrophic denitrifying bacteria.

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