Electron Transfer from Quinohemoprotein Alcohol Dehydrogenase to Blue Copper Protein Azurin in the Alcohol Oxidase Respiratory Chain of *Pseudomonas putida* HK5[†]

Kazunobu Matsushita,* Tetsuo Yamashita, Nahoko Aoki, Hirohide Toyama, and Osao Adachi

Department of Biological Chemistry, Faculty of Agriculture, Yamaguchi University, Yamaguchi, Yamaguchi 753-85l5, Japan

Received January 20, 1999; Revised Manuscript Received March 8, 1999

ABSTRACT: A blue copper protein was purified together with a type II quinohemoprotein alcohol dehydrogenase (ADH IIB) from the soluble fraction of *Pseudomonas putida* HK5 grown on *n*-butanol. The purified blue copper protein was shown to be azurin, on the basis of several properties such as its absorption maximum (623 nm), its low molecular mass (17 500 Da), its acidic nature (p*I* of 4.1), its relatively high redox potential (306 mV), the presence of an intramolecular disulfide bond, and N-terminal amino acid sequence homology with respect to azurins from other sources, especially from *P. putida* NCIB 9869 and *Pseudomonas fluorescens*. Direct electron transfer from ADH IIB to azurin was shown to occur at a rate of 48−70 s^{-1}. The apparent K_m value of ADH IIB for azurin, determined by steady-state kinetics, was decreased several-fold by increasing the ionic strength. Furthermore, the extent of fluorescence quenching of ADH IIB due to the interaction with azurin was increased by increasing the ionic strength, but the binding constant for binding between ADH IIB and azurin was unchanged. The redox potential of azurin was increased 12 mV by incubation with ADH but not vice versa. Furthermore, the redox potential gap between ADH and azurin was increased from 102 to 126 mV by increasing the ionic strength. It is conceivable that a hydrophobic interaction is involved in the electron transfer between both proteins, and it is also suggested that the electron transfer may occur by a freely reversible on and off binding process but may not be related to the global binding process of both proteins. Thus, the results presented here strongly suggest that azurin works as an electron-transfer mediator in a PQQ-dependent alcohol oxidase respiratory chain in *P. putida* HK5.

The alcohol oxidase respiratory chain is exploited in several aerobic oxidative bacteria such as methylotrophs, pseudomonads, or acetic acid bacteria, where quinoprotein alcohol dehydrogenase (ADH)[1] works in periplasm in addition to cytoplasmic NAD-dependent alcohol dehydrogenase. Methanol dehydrogenase (MDH), present in methylotrophic bacteria, was the first quinoprotein shown to have pyrroloquinoline quinone (PQQ) as the prosthetic group (*1*). Thereafter, other ADHs present in oxidative but nonmethylotrophic bacteria have been also shown to be quinoproteins, and to be classified into three groups (types I−III) according to their molecular properties, catalytic properties, and localization (*2*). Type I ADH (ADH I) has been found in *Pseudomonas aeruginosa* (*3*−*5*) and *Pseudomonas putida* (*6*). This ADH donates electrons to an artificial electron acceptor, phenazin methosulfate, in the presence of amine or ammonia. ADH I resembles methanol dehydrogenase with respect to its molecular properties, but not with respect to its affinity for alcohol; it has a much higher affinity for ethanol than for methanol. Thus, it is also known as EDH (*3*−*5*). Type II ADH (ADH II) contains heme *c* besides PQQ and thus is called quinohemoprotein ADH. It has been found in *Comamonas testosteroni* (*7*, *8*) and *P. putida* (*6*). Unlike ADH I, this type of ADH can donate electrons not only to phenazin methosulfate but also to potassium ferricyanide. Type III ADH (ADH III) is a membrane-bound enzyme found in the cytoplasmic membrane of acetic acid bacteria (*2*). It is a complex of three different subunits: a quinohemoprotein dehydrogenase, a triheme cytochrome *c* subunit, and a third subunit whose function is unknown (*9*).

Physiological electron acceptors for some of these ADHs have been studied. It has been shown that MDH reacts with soluble cytochrome c_L, which may transfer electrons to the terminal oxidase via cytochrome c_H (*1*). Type I ADH of *P. aeruginosa* has also shown to react with soluble cytochrome c_{550} (*10*, *11*). Type III ADH has been shown to donate electrons directly to ubiquinone via membrane-bound subunit II, and then to the terminal oxidase (*9*). However, physiological electron acceptors of type II ADH have not been reported to date.

There are two other types of quinoproteins having tryptophan tryptophyl quinone (TTQ) as the prosthetic group: methylamine dehydrogenase (MADH) (*12*) and aromatic amine dehydrogenase (AADH) (*13*). In these systems, two different kinds of copper proteins, amicyanin and azurin, have

[†] This work was supported in part by a grant-in-aid for scientific research from the Ministry of Education, Science, and Culture, Japan (Grants 09044228 and 10660091 to K.M.).

* Corresponding author.

[1] Abbreviations: AADH, aromatic amine dehydrogenase; ADH, alcohol dehydrogenase; ADH I, type I ADH; ADH II, type II ADH; ADH III, type III ADH; DTT, dithiothreitol; MADH, methylamine dehydrogenase; MDH, methanol dehydrogenase; PAGE, polyacrylamide gel electrophoresis; PMS, phenazine methosulfate; PQQ, pyrroloquinoline quinone; TTQ, tryptophan tryptophyl quinone; SDS, sodium dodecyl sulfate.

10.1021/bi990121f CCC: $18.00
Published on Web 04/20/1999

been shown to function as the physiological electron acceptor for MADH of *Paracoccus denitrificans* (*14*) and AADH of *Alcaligenes faecalis* (*15*), respectively. However, the interaction between these quinoproteins and copper proteins has also been shown to be somewhat different; the extent of interaction between MADH and amicyanin increases at high ionic strengths (*16*), while the apparent K_m of AADH for azurin decreases with increasing ionic strengths (*17*). Later, the interaction between MADH and amicyanin was shown to be stabilized by a combination of ionic and van der Waals interactions (*18*).

P. putida HK5 produces three different quinoprotein ADHs, one type I ADH and two type II ADHs (ADH IIB and ADH IIG), which are induced when the organism is grown on ethanol, *n*-butanol, and 1,2-propanediol, respectively (*6*). In this study, a blue copper protein, azurin, was found in *P. putida* HK5 and purified from the soluble fraction of the organism grown on *n*-butanol. This paper describes the molecular properties of the purified azurin and the interaction with type II ADH (ADH IIB). Results clearly indicate that ADH IIB is able to donate electrons to azurin directly, the rate but not the binding of which increases at high ionic strengths.

MATERIALS AND METHODS

Chemicals. All chemicals used in this study were commercial products of guaranteed grade. Azurin of *P. aeruginosa* was purchased from Sigma. Special care was taken to remove traces of alcohol in chemicals known to contain some alcohol used as a dehydrant in chemical manufacturing. In preparation of buffer solutions and ingredients that were used for measurements of ADH activity and also for purification of ADH IIB and azurin, such alcohol was removed by evaporation at 90 °C under reduced pressure.

Bacterial Strain and Growth Conditions. P. putida HK5 (*6*) was grown at 30 °C while it was being shaken (200 rpm) or with a jar fermentor up to the late logarithmic phase in a minimum medium, which is the same as the medium for *P. aeruginosa*, described in a previous paper (*9*). *n*-Butanol was added separately as the sole carbon source to a final concentration of 0.3%. The bacterial growth was monitored with a Klett-Summerson colorimeter.

Preparation of the Soluble Fraction. Cells were collected by centrifugation and washed twice with ice-cold distilled water, and then once with 50 mM potassium phosphate buffer (pH 7.0). The washed cells were resuspended at a concentration of about 1 g of wet cells per 20 mL in the same buffer and incubated while they were shaken for 2 h at 30 °C for consumption of intracellular substrate for ADHs. The cells were harvested once more, washed twice with 50 mM Tris-HCl buffer (pH 8.0), and then resuspended in the same buffer at a concentration of about 1 g of wet cells per 4 mL. Thereafter, a soluble fraction was prepared by disruption of the cells through a French press, followed by two-step centrifugations, as described previously (*6*).

Purification of ADH IIB and Azurin from the Soluble Fraction. The purification procedure for ADH IIB described previously (*6*) was improved as follows. The soluble fraction prepared from *n*-butanol-grown cells was subjected to DEAE-cellulose column chromatography as described previously (*6*). Neither ADH IIB nor azurin was adsorbed by the column, and thus, both were eluted with 50 mM Tris-HCl buffer (pH 8.0), with ADH IIB (pink fraction) eluting before azurin (blue fraction), the latter eluting in the pass-through fraction. To the active ADH IIB fraction was added ammonium sulfate to 30% saturation. After centrifugation to remove precipitated proteins, the resulting supernatant containing ADH IIB was applied to a Butyl-Toyopearl column (about 1 mL of bed volume per 12 mg of protein) which had been equilibrated with 50 mM Tris-HCl buffer (pH 8.0) containing 30% ammonium sulfate. The enzyme was eluted first with 50 mM Tris-HCl buffer (pH 8.0) containing 20% ammonium sulfate, and then with a negative linear gradient of the same buffer system to 10 mM Tris-HCl buffer (pH 8.5). Then the enzymatically active fraction, eluted with 50 mM Tris-HCl buffer (pH 8.0) containing 20% ammonium sulfate, was collected, concentrated by ultrafiltration, dialyzed with 10 mM Tris-HCl buffer (pH 8.5), and then applied to a DEAE-Toyopearl column (about 1 mL of bed volume per 3 mg of protein) pre-equilibrated with the same buffer. The enzyme was eluted with a linear gradient of the same buffer to 50 mM Tris-HCl buffer (pH 8.0). The active fractions were collected, concentrated by ultrafiltration, and then applied to a Superdex S-200 column. Then the enzyme was eluted with 50 mM Tris-HCl buffer (pH 8.0). The fractions absorbing at both 280 and 420 nm were pooled and used as the purified enzyme.

To the azurin fraction separated from the ADH IIB fraction by DEAE-cellulose column chromatography was added ammonium sulfate to 80% saturation. After centrifugation to remove a small amount of ADH IIB together with other impurities, the resulting supernatant containing azurin was applied to a Butyl-Toyopearl column (about 1 mL of bed volume per 6 mg of protein) pre-equilibrated with 50 mM Tris-HCl buffer (pH 8.0) containing 80% ammonium sulfate. The protein was eluted with 10 mM Tris-HCl buffer (pH 8.5) after a stepwise elution with 50 mM Tris-HCl (pH 8.0) containing 20% ammonium sulfate to remove a small amount of ADH IIB. Then the fractions exhibiting absorption at 620 nm were collected, concentrated by ultrafiltration, and dialyzed with 10 mM Tris-HCl buffer (pH 8.5). The protein solution was applied to a DEAE-Toyopearl column (about 1 mL of bed volume per 3 mg of protein) pre-equilibrated with the same buffer, and then eluted with a linear gradient of the same buffer to 50 mM Tris-HCl buffer (pH 8.0). Azurin fractions were collected as in the case of the previous column and then concentrated by ultrafiltration, and applied to a Superdex S-200 column. Then the protein was eluted with 50 mM Tris-HCl buffer (pH 8.0). The fractions absorbing at both 280 and 620 nm were pooled and used as the purified azurin.

Enzyme Assays and Steady-State Kinetics. All enzyme assays were performed at 25 °C as follows. Ferricyanide reductase activity of ADH IIB was measured spectrophotometrically by monitoring the reduction of ferricyanide at 420 nm in a reaction mixture (total of 1 mL) containing 50 mM Tris-HCl buffer (pH 8.0), 1 mM potassium ferricyanide, and an enzyme solution. The reaction was started by adding *n*-butanol at a final concentration of 1 mM. One unit of enzyme activity was defined as the amount of enzyme reducing 1 μmol of ferricyanide per minute, calculated from a millimolar extinction coefficient of potassium ferricyanide of 1.0 mM^{-1} cm^{-1}.

Azurin reductase activity was also measured spectrophotometrically by monitoring the reduction of oxidized azurin at 620 nm in a reaction mixture (total volume of 100 μL) containing various concentrations of Tris-HCl buffer (pH 8.0), various concentrations of azurin, and enzyme solution. The reaction was started by adding *n*-butanol at a final concentration of 1 mM. One unit of the activity was also defined as the amount of enzyme reducing 1 μmol of azurin per minute, calculated from a millimolar extinction coefficient of *P. putida* azurin of 3.38 mM^{-1} cm^{-1} (see below). To determine steady-state kinetic parameters, the azurin reduction rates were measured at different concentrations of azurin, and those data were plotted in double-reciprocal plots.

Analytical Procedures. Polyacrylamide gel electrophoresis (PAGE) in the presence or absence of sodium dodecyl sulfate (SDS) was performed with a 12.5 or 7.5% acrylamide gel, respectively, as described previously (*6*, *9*). Isoelectric focusing was carried out in 5% acrylamide containing 5% Ampholine (pH 3.5−10) (Pharmacia), as described previously (*6*, *9*). N-terminal sequencing of the purified azurin was performed by using the polyvinylidene difluoride microporous membrane to which the protein was blotted from SDS−PAGE, as described previously (*9*). Redox titrations were performed in an anaerobic vessel with a combined platinum electrode (Radiomater, Copenhagen, Denmark) (*19*). Titration was carried out in 30 mM Tris-HCl buffer (pH 7.0 or 8.0) with or without 0.2 M KCl at 25 °C in the presence of the several electron mediators, as described previously (*20*). The course of reduction of heme *c* (ADH IIB) and copper (azurin) was recorded at the α-band maximum at 553 and 620 nm, respectively, using a Hitachi 557 dual-wavelength spectrophotometer. Fluorescence spectra were recorded for 1 mL samples (the concentration of ADH IIB being 0.1 μM and that of azurin being 1.0 μM) in 30 mM Tris-HCl buffer (pH 8.0) with a Hitachi 650-10 S fluorescence spectrophotometer. Fluorescence emission spectra were scanned from 290 to 400 nm at 25 °C with excitation at 275 nm. Absorption spectrophotometry was performed with a Hitachi 557 dual-wavelength spectrophotometer. The copper content of azurin was measured by atomic absorption using the internal standard method with $CuCl_2$. Ultrafiltration using an ARTKISS molecular weight cutoff of 10 000 (Advantec TOYO) was performed according to the published method (*21*). The protein content was determined by the modified Lowry method (*22*).

RESULTS

Purification of ADH IIB and the Blue Copper Protein (Azurin). Both ADH IIB and a blue copper protein were purified from the soluble fraction of the cells grown on *n*-butanol, both proteins being synthesized most effectively under these culture conditions. Although ADH IIB has been purified previously (*6*), the purification procedure was improved in this study, where two ADH IIB fractions, one having a high specific activity and the other having a low specific activity, were separated by Butyl-Toyopearl column chromatography. The former enzyme was eluted with 50 mM Tris-HCl buffer (pH 8.0) containing 20% ammonium sulfate, while the latter was eluted with 10 mM Tris-HCl (pH 8.5). The former was further purified, exhibiting a final specific activity of 22.2 units/mg of protein, and used in all subsequent experiments. No further analysis was done in this study on the latter ADH IIB fraction with the lower specific activity.

For the purification of the blue copper protein, special care was taken to remove traces of alcohol from the buffer solution used and also from the cell extract to keep the protein in the oxidized state so that it could be easily detected as blue fractions. Furthermore, after Butyl- and DEAE-Toyopearl column chromatography, a small amount of ferricyanide was added to each fraction to enable the blue copper protein to be detected at 620 nm for further purification. In the final purification step with Superdex S-200 column chromatography, the blue copper protein was eluted as a symmetrical peak at both 280 and 620 nm.

Spectral Property of ADH IIB. Enzymatic and molecular properties of ADH IIB have been described previously (*6*). In this study, to obtain the exact molar extinction coefficient of the cytochrome *c* in the enzyme, the absolute spectrum and the spectrum for the pyridine hemochrome were determined using the purified enzyme. The enzyme exhibited absorption maxima at 552.5, 524, and 418 nm in the reduced state. On the basis of the heme *c* content (11.2 nmol/mg of protein), the molecular extinction coefficient was estimated to be 32.8, 24.7, and 208.6 mM^{-1} cm^{-1} at the peaks of 552.5, 524, and 418 nm, respectively.

Molecular Properties of the Blue Copper Protein (Azurin). The purified blue copper protein exhibited a single band with a molecular weight of 17 500 on SDS−PAGE. Without dithiothreitol (DTT) treatment, however, it exhibited two bands, one moving a little faster than that with DTT and a second slow-moving weak band, the molecular weights of which were estimated to be 15 500 and 31 000, respectively (Figure 1A). The same phenomenon was seen in a commercial azurin of *P. aeruginosa*, but a higher DTT concentration was required to exhibit a single band in *P. putida* azurin. The two bands of the purified blue copper protein were also seen on native PAGE and on isoelectric focusing PAGE (Figure 1B). These results are consistent with the previous finding (*23*) that azurin has an intramolecular disulfide bond and can also exist as a dimer having an intermolecular disulfide bond. Incidentally, the isoelectric points of the intramolecular and intermolecular disulfide forms were determined to be 4.1 and 3.6, respectively (Figure 1C).

The purified blue copper protein exhibited absorption maxima at 276 and 623 nm in the oxidized state (Figure 2), which are comparable to the maxima at 280 and 630 nm of azurin from *P. aeruginosa* (*24*). However, the absorption derived from aromatic amino acids (276 nm) relative to that derived from copper (623 nm) was extremely low in the blue copper protein purified from *P. putida*. In fact, the protein content of the *P. putida* blue copper protein was significantly underestimated by the Lowry method (data not shown). Thus, the millimolar absorption coefficient of the *P. putida* blue copper protein was estimated to be 3.38 at 623 nm, on the basis of the copper content that was determined by atomic absorption of the purified protein, not on the basis of the protein content. This value was actually very low compared with that, 6.95 at 630 nm, of *P. aeruginosa* azurin (*24*).

The 19 N-terminal amino acids of the purified blue copper protein from *P. putida* HK5 were identified with a peptide sequence analyzer, and compared with sequences from various azurins from different sources (Figure 3). The

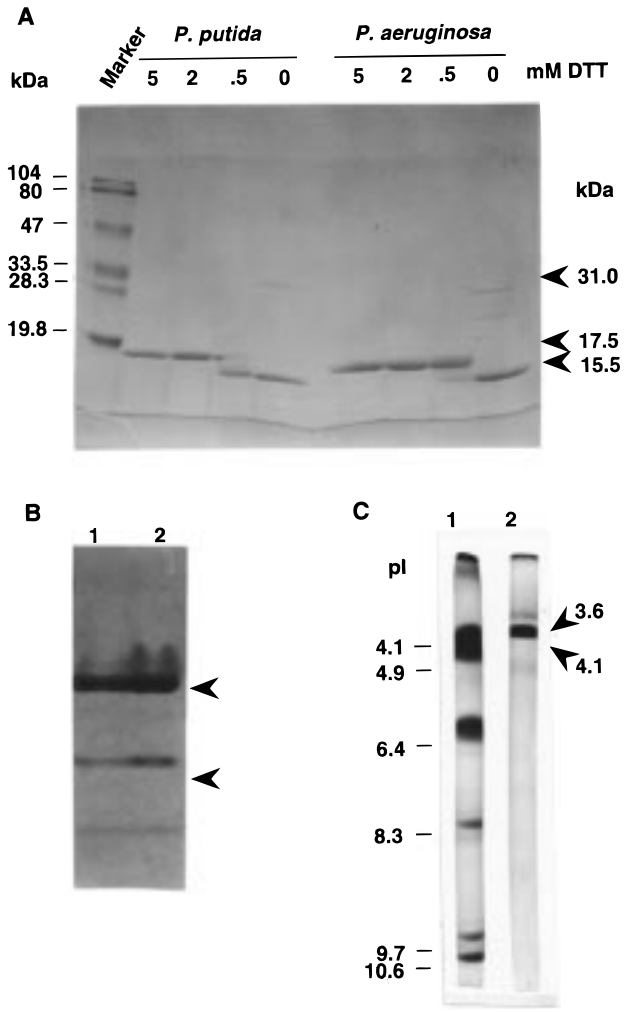

FIGURE 1: SDS−PAGE, native PAGE, and isoelectric focusing PAGE of azurin. (A) Azurin (5 μg of protein) purified from *P. putida* HK5 and commercial azurin of *P. aeruginosa* (Sigma) were treated with a sample buffer containing various concentrations (0, 0.5, 2, and 5 mM) of DTT and then subjected to SDS−PAGE. Prestained marker proteins (Bio-Rad) was used as molecular size standards. (B) Purified azurin (lanes 1 and 2, 5 and 10 μg of protein, respectively) was analyzed by native PAGE. (C) Marker proteins (lane 1, 60 μg of acetylated cytochromes *c*) and purified azurin (lane 2, 20 μg of protein) were analyzed by isoelectric focusing PAGE.

sequence of the blue copper protein of *P. putida* HK5 is very similar to the sequences of two azurins isolated from *P. fluorescens* biotype B (*25*) and *P. putida* NCIB 9869 (*26*), but relatively slightly similar to the sequences of azurins isolated from *P. aeruginosa* (*27*) and *Alcaligenes denitrificans* NCTC 8582 (*27*).

Reactivity of the Blue Copper Protein (Azurin) with ADH IIB. As described in the purification section, the blue copper protein could be present in a reduced state in the crude extract or in the crude enzyme solution. This seems to be due to the presence of ADH IIB and a trace amount of alcohol in the solutions, since depletion of alcohol from the buffer solution produced the protein oxidized state. Since the blue copper protein seemed to be reduced by the electron transfer activity of ADH IIB, the reactivity of the blue copper protein with ADH IIB was measured and compared with the ferricyanide reductase activity. The blue copper protein reductase activity of ADH IIB was observed over a relatively broad pH ranging from 6.5 to 8.5, the optimum pH being

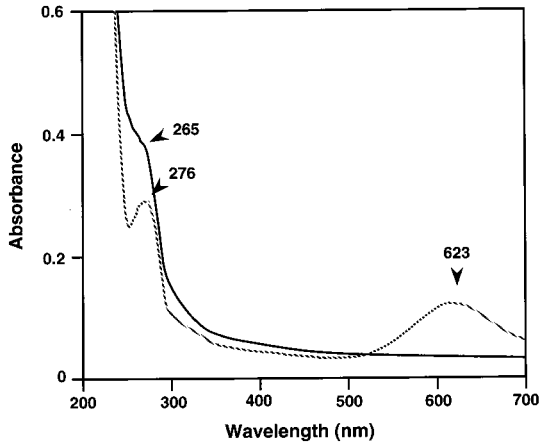

FIGURE 2: Absorption spectra of purified azurin from *P. putida* HK5. The samples were preoxidized with a small amount of ferricyanide and then dialyzed against 50 mM Tris-HCl (pH 8.0). Absorption spectra were measured at room temperature with the dialysates (0.46 mg/mL, broken line) and then with the same samples reduced with $NaBH_4$ (solid line).

strains	N-terminal sequence
P. putida HK5	1:**ADXKVTVDSTDQMSFNTKA**:19
P. putida (NCIB 9869)	1:**AECKVTVDSTDQMSFNTK**D:19
P. fluorescens biotype B	1:**AECKTTIDSTDQMSFNTKA**:19
A. denitrificans (NCTC 8582)	21:**AQCEATIESNDAMQYNLKE**:39
P. aeruginosa	21:**AECSVDIQGNDQMQFNTNA**:39
	* * * * *

FIGURE 3: Alignment of the N-terminal amino acid sequence of azurin of *P. putida* HK5 with azurins from other microorganisms. The sequence number of amino acids in azurins of *A. denitrificans* and *P. aeruginosa* is based on the sequences including the signal sequence. The amino acids that are identical to those of azurin of *P. putida* HK5 are shown with bold letters, and those conserved in all proteins are marked with an asterisk.

Table 1: Kinetic Constants of ADH IIB for Ferricyanide and Azurins of *P. putida* and *P. aeruginosa*[a]

electron acceptor	buffer	K_m (μM)	V_{max} (units/mg)	k_{cat} (s^{-1})
azurin (Pp[b])	30 mM Tris-HCl (pH 8.0)	100	62.5	71.8
	30 mM Tris-HCl and 0.1 M KCl	51	52.6	60.5
	30 mM Tris-HCl and 0.5 M KCl	25	41.7	48.0
azurin (Pa[b])	30 mM Tris-HCl (pH 8.0)	202	22.3	25.6
ferricyanide	30 mM Tris-HCl (pH 8.0)	95	45.5	52.3

[a] Azurin reductase activity of ADH IIB was measured in 30 mM Tris-HCl buffer (pH 8.0) containing various concentrations of KCl. Apparent K_m and V_{max} values were determined from double-reciprocal plots. [b] Azurin Pp and Pa represent azurin from *P. putida* HK5 and *P. aeruginosa*, respectively.

8.0. As shown in Table 1, the reduction activity of ADH IIB toward the blue copper protein (62.5 units/mg) was higher than that of the ferricyanide reductase activity (45.5 units/mg). In contrast, the reductase activity of ADH IIB was rather low toward *P. aeruginosa* azurin. When measured at a concentration of 15 μM, the reductase activity of the blue copper protein was increased 2.0-fold when the ionic strength was increased and saturated at a KCl concentration of around 0.25 M (Figure 4). Kinetic analysis showed that the K_m values of ADH IIB for the blue copper protein decreased with increasing ionic strengths (Figure 4 and Table 1), indicating that the affinity of ADH IIB for the *P. putida* blue copper protein increases with increasing ionic strengths.

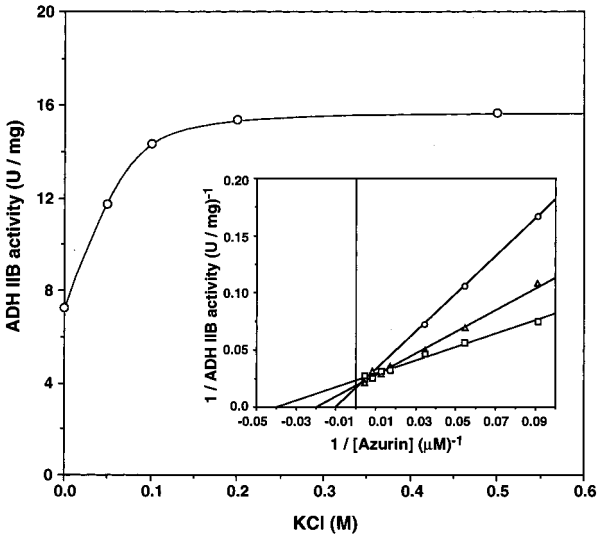

FIGURE 4: Reduction kinetics of azurin purified from *P. putida* HK5 with ADH IIB at different ionic strengths. Azurin reductase activity was measured with 15 μM azurin in 30 mM Tris-HCl (pH 8.0) containing various concentrations of KCl. In the inset is shown a reciprocal plot of azurin reductase activity with various concentrations of azurin. The activity was measured in 30 mM Tris-HCl (pH 8.0) containing 0 (○), 0.1 (△), and 0.5 M KCl (□).

Redox Potential of the Blue Copper Protein (Azurin) and ADH IIB. Redox titration of heme *c* in ADH IIB and of the copper site in the blue copper protein were carried out at different ionic strengths (Figure 5). At first, redox titrations of ADH IIB and the blue copper protein were carried out at pH 7.0, the standard pH for redox titration, where the redox potentials of heme *c* of ADH IIB and copper of the blue copper protein were determined to be 185 and 306 mV, respectively (data not shown). Next, redox titrations of ADH IIB, the blue copper protein, and the mixture were carried out at pH 8.0, the optimum pH of the blue copper protein reductase activity of ADH IIB. In 30 mM Tris-HCl, ADH IIB and the blue copper protein separately exhibited redox potentials of 188 and 280 mV, respectively (Figure 5A), while together, the potentials were 189 and 291 mV, respectively (Figure 5B). In the presence of 0.3 M KCl, the redox potentials of free ADH IIB and free blue copper protein were 172 and 286 mV (Figure 5C), respectively, while in the mixture, they were 172 and 298 mV, respectively (Figure 5D). These results indicated that the redox potential of the heme *c* component in ADH IIB is not changed by the presence of the blue copper protein, but dropped by about 16 mV at high ionic strengths (from 188 to 172 mV or from 189 to 172 mV in the absence or presence, respectively, of the blue copper protein). On the other hand, the redox potential of the copper center in the blue copper protein increases by about 10 mV in the presence of ADH IIB at all ionic strengths (from 280 to 291 mV or from 286 to 298 mV without or with, respectively, 0.3 M KCl), but undergoes a small change in potential (6−7 mV) with the change in ionic strength, which is smaller than the change in that of ADH IIB due to ionic strength (−16 mV).

Interaction of the Blue Copper Protein (Azurin) with ADH IIB. To detect the interaction between the blue copper protein and ADH IIB, fluorescence measurement was performed. First, fluorescence emission spectra of oxidized ADH IIB (0.1 μM), the blue copper protein (1.0 μM), and the mixture were recorded (Figure 6). The blue copper protein exhibited

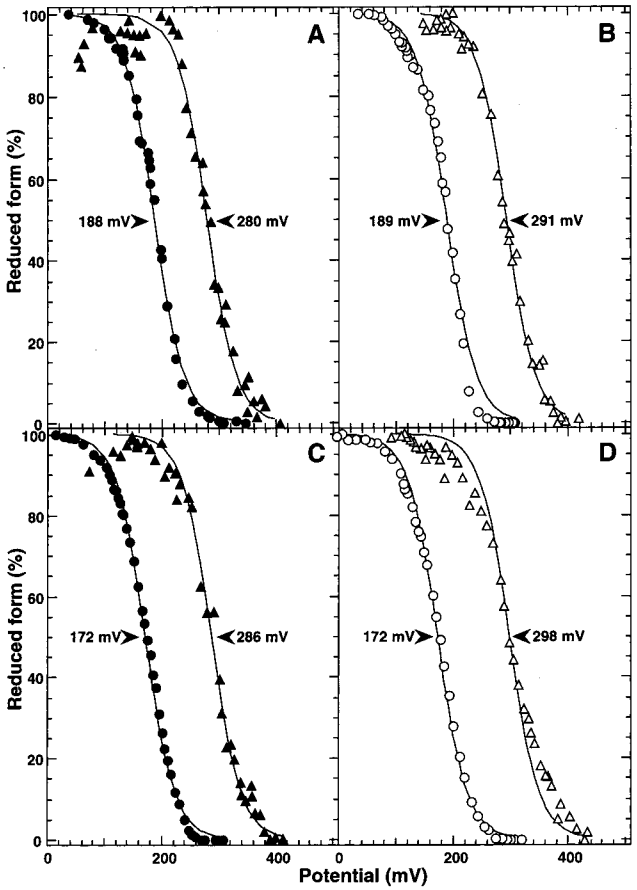

FIGURE 5: Potentiometric titration of heme *c* in ADH IIB and the copper center in azurin. Redox titration was carried out at 25 °C, as described in Materials and Methods, with 10 μM azurin or 10 μM ADH IIB separately [panels A and C, (●) ADH IIB and (▲) azurin] and together [panels B and D, (○) ADH IIB and (△) azurin] in 30 mM Tris-HCl (pH 8.0) (panels A and B) and in the same buffer containing 0.3 M KCl (panels C and D). The plots represent data from eight independent titrations in both oxidative and reductive directions. Experimental data in all panels were fitted by the Nernst curve for one component ($n = 1$) with unknown redox potentials using Igor Pro software for Macintosh (Wave Metrics, Lake Oswego, OR). All potentials are relative to the standard hydrogen electrode.

almost no intrinsic fluorescence from tryptophan, which is consistent with the lack of absorption near the UV region (Figure 2). In contrast, ADH IIB exhibited a relatively high-level fluorescence emission spectrum, the maximum of which was around 337 nm. The fluorescence at 337 nm was somewhat higher in the oxidized than in the reduced state (data not shown), and was quenched slightly by increasing the ionic strength (data not shown).

As shown in Figure 6, when oxidized ADH IIB and oxidized blue copper protein were mixed, curve d was obtained. When curve d was compared with curve e (the sum of curves b and c from the separate proteins), the fluorescence of ADH IIB was seen to be decreased by the addition of the blue copper protein. The extent of fluorescence quenching of ADH IIB due to the blue copper protein was plotted as the saturation curve (Figure 7A) and also as reciprocal plots (Figure 7B) to obtain a measure of binding of the two proteins. Although the extents of fluorescence quenching at low blue copper protein concentrations deviate from linearity (Figure 7B), this is probably due to the concentration of azurin being too close to that of ADH IIB

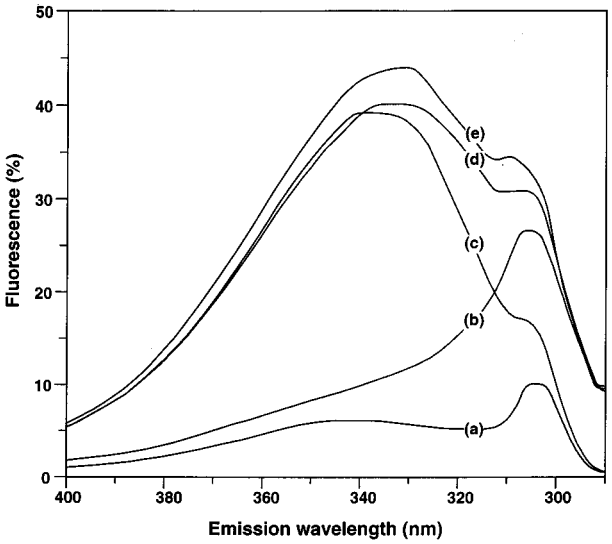

FIGURE 6: Fluorescence emission spectra of free ADH IIB, azurin, and the mixture. Spectra were recorded at 25 °C in a 1 mL reaction mixture containing 30 mM Tris-HCl (pH 8.0) with excitation at 275 nm. These proteins were preoxidized by ferricyanide and dialyzed against the same buffer before being used: (a) buffer control, (b) 1.0 μM azurin, (c) 0.1 μM ADH IIB, (d) a mixture of 1.0 μM azurin and 0.1 μM ADH IIB, and (e) theoretical curve for the mixture obtained from curves b and c.

(0.1 μM). As shown in the figures, the K_d value for binding of the blue copper protein was determined to be 0.24 μM at all ionic strengths. Although the ionic strength did not affect the K_d values, the extent of fluorescence quenching was increased by increasing ionic strengths.

Although the data are not shown here, no complex forms of ADH IIB and the blue copper protein were observed on HPLC gel filtration using Superdex S-200, or on ultrafiltration using a membrane filter.

DISCUSSION

In this study, a small blue copper protein was purified, together with quinohemoprotein ADH IIB, from the soluble fraction of *P. putida* HK5 grown on *n*-butanol. The blue copper protein exhibits several characteristics similar to those of other bacterial blue copper protein such as amicyanin, azurin, and pseudoazurin, as follows. (i) The purified protein exhibits an absorption maximum at 623 nm. (ii) The purified protein has a low molecular mass (17 500 Da). (iii) The protein is acidic (p*I* of 4.1). (iv) The protein has a redox potential of 306 mV at pH 7.0. (v) The protein may have an intramolecular disulfide bond. (vi) The N-terminal amino acid sequence of the purified protein is closely homologous to those of azurins from other sources. As judged from the above criteria, especially from the sequence homology, the blue copper protein isolated from *P. putida* HK5 is thought to be azurin.

Azurin has been found to be a redox protein working in the periplasm of several Gram-negative bacteria such as some pseudomonads, some methylotrophs, and *Alcaligenes* species. It has been shown by in vitro study to work as an electron donor or acceptor for nitrite reduction in some pseudomonads (*28*, *29*), although it has been shown by in vivo study not to be necessarily required for denitrification of *P. aeruginosa* (*30*). Thus, azurin could be produced under some conditions where the anaerobic nitrate−nitrite reduction respiratory chain is exploited. However, this is not the case for *P. putida* HK5 which was used in this study because this strain has been shown to have no ability to achieve denitrification (*6*).

A blue copper protein is also found in the aerobic respiratory chain. Amicyanin or azurin has been shown to work as an electron acceptor for TTQ-dependent quinoproteins, MADH and AADH, respectively, of *Pa. denitrificans* (*14*) and of *A. faecalis* (*15*). More recently, a blue copper protein has been shown to mediate an electron transfer from quinoprotein butylamine dehydrogenase to the membrane respiratory chain of *P. putida* IFO15366 (*31*). Amicyanin or azurin is able to react directly with the quinoprotein amine dehydrogenases, and thus works as an electron transfer mediator in the aerobic amine oxidase respiratory chain.

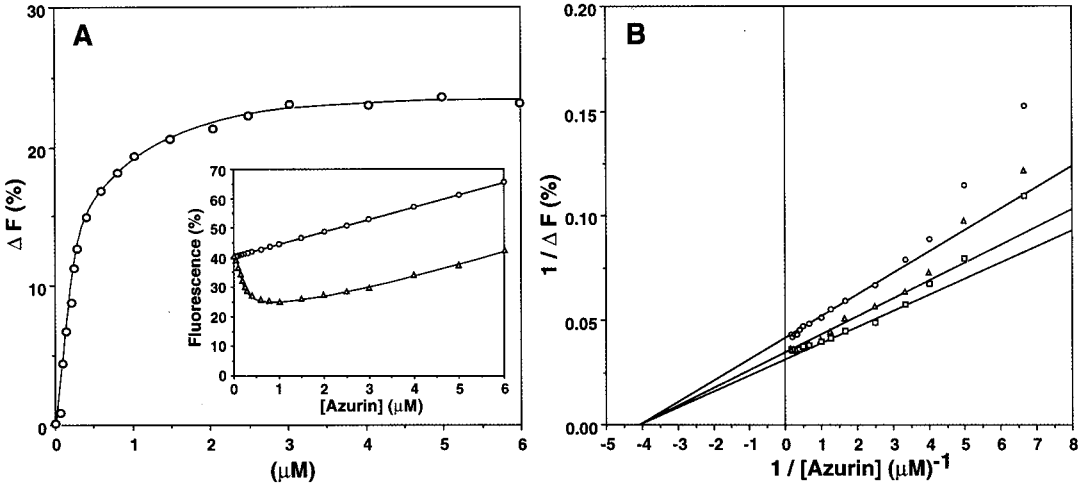

FIGURE 7: Fluorescence quenching of ADH IIB with various concentrations of azurin. The fluorescence was recorded at 25 °C in a 1 mL reaction mixture containing 30 mM Tris-HCl (pH 8.0) and 0.1 μM ADH IIB. Azurin was added from a stock solution of 118.5 μM azurin and 0.1 μM ADH IIB to give the desired final concentration. (A) The extents of quenching (ΔF) were values of the difference between the theoretical values and the experimental values as shown in the inset, and plotted against the azurin concentration. In the inset is shown the fluorescence of ADH IIB in the presence of various concentrations of azurin: (○) theoretical line calculated from the fluorescence at 337 nm of various concentrations of azurin and that of 0.1 μM ADH IIB and (△) experimental data of the fluorescence at 337 nm of ADH IIB in the presence of azurin. (B) The reciprocal plot of quenching of ADH IIB for azurin at different ionic strengths. The reaction was performed as described for panel A in 30 mM Tris-HCl (pH 8.0) (○) and in the same buffer containing 0.1 (△) or 0.3 M KCl (□).

PQQ-dependent alcohol oxidase respiratory chains have been found in methylotrophs, *P. aeruginosa*, and *P. putida* HK5, the latter two species of which have been recently shown to contain azurin when grown on alcohol. In *P. aeruginosa*, azurin has been shown to mediate an electron transfer from type I ADH via a cytochrome c_{551} to a terminal cytochrome *co*-type oxidase (S. DeVeries et al., personal communication). This situation could be very similar to that of the methylotroph, *Methylomonas* sp. strain J, where azurin (azurin iso-1) has been shown to accept electrons from cytochrome *c* (I) reduced by MDH (*32*, *33*). In this study, type II ADH (ADH IIB) was also shown to directly react with azurin, which can be assessed by steady-state kinetics of butanol-dependent reduction of azurin by ADH IIB. The V_{max} value of the electron transfer rate for transfer from ADH IIB to azurin corresponds to a k_{cat} of 72 or 48 s^{-1} in the absence or presence of 0.5 M KCl. The electron transfer rate is reasonably high compared to the estimated physiological rate of ADH IIB since the turnover rate in the intact cells is calculated to be 2−8 s^{-1} on the basis of both the ADH IIB activity and alcohol oxidation activity of the resting cells (K. Matsushita et al., unpublished data).

The results of this study also indicate that there is an interaction between ADH IIB and azurin which is dependent on their hydrophobic nature. This is supported by the findings that the affinity of ADH IIB for azurin was shown by steady-state kinetics to increase with increasing ionic strengths, and also that the extent of fluorescence quenching of ADH IIB by interacting with azurin increased with increasing ionic strengths. Such a hydrophobic interaction between ADH IIB and azurin seems to mediate the electron transfer reaction, which is also favored by higher ionic strengths. This is a situation similar to the case of AADH and azurin (*16*) but different from the case of MADH and amicyanin where the electron transfer reaction is inhibited at higher ionic strengths (*17*).

The redox titration carried out in this study also has given us some information about the interaction between ADH IIB and azurin. The redox potential of ADH was decreased 16 mV in the presence of high salt, but that of azurin was increased only a little (6 mV). However, mixing the two proteins increased the redox potential of azurin by 12 mV but left unchanged that of ADH IIB. Thus, in the presence of high salt, the redox potential gap between the two proteins is increased from 102 to 126 mV, which is thermodynamically reasonable in view of the increased electron transfer rate for transfer from ADH IIB to azurin. Furthermore, from the titration data, two possibilities for conformational changes in both proteins exist. (i) The change in the redox potential of azurin in the presence of ADH IIB may result from some conformational change in azurin upon interaction with ADH. (ii) The change in the redox potential of ADH IIB in the presence of high salt suggests that ADH IIB may undergo some conformational change near the heme *c* site, which may stimulate the electron transfer to azurin. This idea is also supported by the finding that the extent of fluorescence quenching of ADH IIB increases a little at high ionic strengths.

However, the binding constant for binding between ADH IIB and azurin was not affected by ionic strength, as indicated by the extent of fluorescence quenching. This is totally different from the case of MADH and amicyanin, where the K_d value is markedly increased, as well as rate of the electron transfer reaction, at higher ionic strengths (*21*). Thus, although the relationship between the electron transfer reaction and the binding process between ADH IIB and azurin seems to be complicated, it may be explained in one of two ways. By the interaction with or the electron transfer from ADH, as suggested above, azurin may undergo a conformational change, which stimulates the dissociation of azurin from ADH. If this is the case, higher ionic strengths would increase the rate of dissociation as well as association of both proteins, leaving the apparent dissociation constant for dissociation of both proteins unchanged, even though the rate of electron transfer is increased, at higher ionic strengths. Another possibility is that there is a binding site between both proteins separated from a site involved in the electron transfer. If the global binding process for binding between both proteins is dependent on the binding site but not on the site for electron transfer, the binding process is not rate-limiting for the electron transfer reaction. Thus, the apparent dissociation constant may be independent of the electron transfer reaction between both proteins. Although it is very hard to distinguish these possibilities, some information could be obtained if a crystal structure of the associated proteins were solved in the future as in the case of MADH and amicyanin (*18*).

Although data are not shown, azurin has been shown to mediate an electron transfer from ADH IIB, which may be freely soluble in the periplasm, to the membrane-bound respiratory chain, probably cytochrome oxidase, of *P. putida* HK5 (K. Matsushita et al., unpublished observation). Furthermore, *P. putida* HK5 expresses another ADH, ADH I, in the periplasm at the same time as ADH IIB (*6*), and by analogy to that of *P. aeruginosa*, ADH I is expected to transfer electrons to azurin via cytochrome *c*. Thus, azurin may work as the electron acceptor for both ADH IIB and the cytochrome *c* in the organism. Therefore, azurin would be most efficient as a freely reversible electron transfer mediator between two or more soluble redox components and the redox component(s) fixed in the membrane. Thus, in the alcohol oxidase respiratory chain of *P. putida* HK5, azurin may transfer electrons to the membrane-bound respiratory component by freely attaching to and also detaching from ADH IIB.

ACKNOWLEDGMENT

We thank Dr. Kenji Kano (Kyoto University, Kyoto, Japan) and Dr. Haruo Suzuki (Kitasato University, Sagamihara, Japan) for critical reading and discussion and Ms. Ryoko Uenishi for technical assistance.

REFERENCES

1. Anthony, C. (1993) Methanol dehydrogenase in Gram-negative bacteria, in *Principles and applications of quinoproteins* (Davidson, V. L., Ed.) pp 17−45, Marcel Dekker, Inc., New York.
2. Matsushita, K., Toyama, H., and Adachi, O. (1994) *Adv. Microbiol. Physiol. 36*, 247−301.
3. Rupp, M., and Gorisch, H. (1993) *Biol. Chem. Hoppe-Seyler 369*, 431−439.
4. Schrover, J. M., Frank, J., van Wielink, J. E., and Duine, J. A. (1993) *Biochem. J. 290*, 123−127.
5. Diehl, A., Wintzingerode, F., and Görisch, H. (1998) *Eur. J. Biochem. 257*, 409−419.

6. Toyama, H., Fujii, A., Matsushita, K., Shinagawa, E., Ameyama, M., and Adachi, O. (1995) *J. Bacteriol. 177*, 2442−2450.
7. Groen, B. W., van Kleef, M. A., and Duine, J. A. (1986) *Biochem. J. 234*, 611−615.
8. Jongejan, A., Jongejan, J. A., and Duine, J. A. (1998) *Protein Eng. 11*, 185−198.
9. Matsushita, K., Yakushi, T., Toyama, H., Shinagawa, E., and Adachi, O. (1996) *J. Biol. Chem. 271*, 4850−4857.
10. Reichman, P., and Görisch, H. (1993) *Biochem. J. 289*, 173−178.
11. Schobert, M., and Görisch, H. (1999) *Microbiology 145*, 471−481.
12. McIntire, W. S., Wemmer, D. E., Chistoserdov, A., and Lidstrom, M. E. (1991) *Science 252*, 817−824.
13. Govindaraj, S., Eisenstein, E., Jones, L. H., Sanders-Loehr, J., Chistoserdov, A. Y., Davidson, V. L., and Edwards, S. L. (1994) *J. Bacteriol. 176*, 2922−2929.
14. Husain, M., and Davidson, V. L. (1985) *J. Biol. Chem. 260*, 14626−14629.
15. Edwards, S. L., Davidson, V. L., Hyun, Y. L., and Wingfield, P. T. (1995) *J. Biol. Chem. 270*, 4293−4298.
16. Hyun, Y. L., and Davidson, V. L. (1995) *Biochemistry 34*, 12249−12254.
17. Davidson, V. L., and Jones, L. H. (1991) *Anal. Chim. Acta 249*, 235−240.
18. Davidson, V. L., Jones, L. H., Graichen, M. E., Mathews, F. S., and Hosler, J. P. (1997) *Biochemistry 36*, 12733−12738.
19. Dutton, P. L. (1978) *Methods Enzymol. 54*, 411−435.
20. Frébortová, J., Matsushita, K., Arata, H., and Adachi, O. (1998) *Biochim. Biophys. Acta 1363*, 24−34.
21. Davidson, V. L., Graichen, M. E., and Jones, L. H. (1993) *Biochim. Biophys. Acta 1144*, 39−45.
22. Dulley, J. R., and Grieve, P. A. (1975) *Anal. Biochem. 64*, 136−141.
23. Bonander, N., Karlsson, B. G., and Vänngård, T. (1995) *Biochim. Biophys. Acta 1251*, 48−54.
24. Sutherland, I. W., and Wilkinson, J. F. (1963) *J. Gen. Microbiol. 30*, 105−112.
25. Ambler, R. P., and Brown, L. H. (1967) *Biochem. J. 104*, 784−825.
26. Barber, M. J., Trimboli, A. J., and McIntire, W. S. (1993) *Arch. Biochem. Biophys. 303*, 22−26.
27. Hoitink, C. W. G., Woudt, L. P., Turenhout, J. C. M., Van de Kamp, M., and Canters, G. W. (1990) *Gene 90*, 15−20.
28. Zumft, W. G., Gotzmann, D. J., and Kroneck, P. M. (1987) *Eur. J. Biochem. 15*, 301−307.
29. van de Kamp, M., Silvestrini, M. C., Brunori, M., Van Beeumen, J., Hali, F. C., and Canters, G. W. (1990) *Eur. J. Biochem. 194*, 109−118.
30. Vijgenboom, E., Busch, J. E., and Canters, G. W. (1997) *Microbiology 143*, 2853−2863.
31. Adachi, O., Kubota, T., Hacisalihoglu, A., Toyama, H., Shinagawa, E., Duine, J. A., and Matsushita, K. (1998) *Biosci., Biotechnol., Biochem. 62*, 469−478.
32. Sumio, O., and Tobari, J. (1981) *J. Biochem. (Tokyo) 90*, 215−224.
33. Taguchi, K., Kudo, T., and Tobari, J. (1998) *Biosci., Biotechnol., Biochem. 62*, 870−874.

BI990121F