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Characterization of Nitrous Oxide Reductase from a Methylotrophic Denitrifying Bacterium, *Hyphomicrobium denitrificans* A3151

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A Cu-containing nitrous oxide reductase (HdN_2OR) from a methylotrophic denitrifying bacterium, *Hyphomicrobium denitrificans* A3151, has been aerobically prepared and spectroscopically characterized. Purple and blue forms of HdN_2OR have been isolated. Each form is a homodimer comprising monomers with a molecular mass of 65 kDa. The visible absorption spectrum of the purple form (designated as form A) exhibits three absorption bands at 480 nm, 540 nm, and 650 nm, with a shoulder near 780 nm, and that of the blue form (designated as form B) shows only one absorption band at 650 nm. Reversible spectral changes, between those of forms A and B, are observed on treatment of these forms with redox reagents. Forms A and B are oxidized and reduced forms, respectively. The 77-K EPR spectrum of form A indicates a seven-line copper hyperfine structure centered at $g_{//}$ ($g_{//} = 2.18$, $A_{//} = 4.5$ mT), which is characteristic of a mixed-valence binuclear Cu_A site (A_{mv}), and that of form B exhibits a broad featureless signal ($g = 2.06$). The various spectral data of HdN_2OR suggest that form A contains A_{mv} and a mixed-valence tetranuclear Cu_Z site (Z_{mv}^*), while form B includes reduced Cu_A (A_{red}) and Z_{mv}^*. The pH profiles of N_2OR activity of the two forms are similar to each other, and the specific activity at optimum pH 8.8 was estimated to be 45 ± 5 and 29 ± 3 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ for forms A and B, respectively.

Key words: binuclear copper center, copper enzyme, denitrification, methylotrophic denitrifying bacterium, nitrous oxide reductase, tetranuclear copper center.

Abbreviations: HdN_2OR, nitrous oxide reductase from *Hyphomicrobium denitrificans* A3151; N_2OR, nitrous oxide reductase.

Denitrification is the dissimilatory reduction of nitrate or nitrite, usually to produce dinitrogen, by prokaryotic organisms, and comprising anaerobic reduction processes ($NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$) that are caused in either the cytoplasmic membrane or periplasm by the corresponding oxidoreducatases containing transition metal ions (*1*). As the last step of these processes, the conversion of N_2O to N_2 is catalyzed by Cu-containing nitrous oxide reductase (N_2OR), which is a functional homodimer comprising monomers with a molecular mass of 65 kDa. Since N_2OR was first isolated from *Pseudomonas stutzeri* (*2*), the enzymes from many denitrifying bacteria have been reported (*3–14*). The X-ray crystal structures of the N_2ORs from *Pseudomonas nautica* (*15*, *16*) and *Paracoccus denitrificans* (*16*, *17*) were recently solved. They each comprise a head-to-tail homodimer and each monomer has two redox-active Cu centers: a binuclear electron input site called Cu_A and a tetranuclear catalytic site termed Cu_Z. The neighboring Cu_A and Cu_Z centers belong to different subunits in the dimeric protein, and *ca.* 10 Å away from each other, while the intrasubunit distance between Cu_A and Cu_Z is *ca.* 40 Å. The electrons are transferred from Cu_A to Cu_Z across the subunit interface. The Cu_A site is spectroscopically and structurally similar to the Cu_A center of cytochrome c oxidase (*18*, *19*). Comparison of the multifrequency EPR spectra of N_2OR and cytochrome c oxidase suggested that in both enzymes the EPR detectable Cu_A site forms a binuclear mixed-valence center (A_{mv}) (*20*). The coupled Cu ions are ligated by two bridging cysteine sulfurs, two histidine nitrogens, one methionine sulfur, and one main chain carbonyl oxygen of glutamate. The distance between the Cu ions in Cu_A is 2.5 Å. X-ray crystal structure analysis revealed that the Cu_Z site catalyzing N_2O reduction is located in the middle of a seven-bladed β-sheet propeller domain. Judging from the result of spectroscopic and crystallographic studies, Cu_Z is a novel tetranuclear Cu cluster shaped like a distorted tetrahedron with seven histidine ligands, three hydroxide or water ligands, and a bridging inorganic sulfide ion (*15–17*, *21*). According to the results of Cu K-edge XAS experiments, the oxidation state of Cu_Z is 1Cu(II)/3Cu(I) and the total spin for the four Cu atoms is 1/2 (*22*).

N_2OR exists in several forms, which are distinguished by their redox and spectroscopic properties and enzymatic activity (*1*, *23*, *24*). The enzyme can be isolated anaerobically as a high-activity "purple" form (previously designated as N_2OR I), but aerobically as a low-activity "pink" form (previously designated as N_2OR II). Both forms exhibit distinctive EPR signals with 7-line hyperfine splitting in the g_z region, which have been attributed to a delocalized unpaired electron in the dinuclear Cu_A site [Cu(1.5+)-Cu(1.5+)]. This mixed-valence site gives a unique absorption spectrum with a prominent peak at

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540 nm, a weak band at 480 nm, and a shoulder at 480 nm. Moreover, the enzyme generally takes on two Cu_Z states showing different redox and spectroscopic properties, which are an oxidized form [Z_{ox}, $E_{1/2}$ = +60 mV (*25*)] and an inactive or blocked mixed-valence form [Z_{mv}^*, $E_{1/2}$ > +400 mV (*25*)] (*26–28*). The "purple" form of N_2OR under anaerobic conditions has mainly diamagnetic Z_{ox} with a small proportion of Z_{mv}^*, while the "pink" form of N_2OR under aerobic conditions has a relatively large proportion of Z_{mv}^* (*28*). The proportion of Z_{mv}^* varies among preparations. One-electron reduction of the high-activity "purple" form with ascorbate under anaerobic conditions produces semireduced N_2OR, in which Cu_A [$E_{1/2}$ = +260 mV (*25*)] is reduced to a spectroscopically silent moiety [A_{red}, Cu(1+)-Cu(1+)], and the remaining absorption bands at 550 and 640 nm are attributed to Z_{ox}. Further one-electron reduction of the enzyme yields "blue" reduced N_2OR (previously called N_2OR III) exhibiting a single absorption band at 650 nm. This feature has been ascribed to the reduced Cu_Z form (Z_{mv}^*) characterized as a mixed-valence cluster, which exhibits a broad EPR signal with 4-line hyperfine splitting in the g_z region (*14*, *28*).

In this paper, we report the isolation, purification, and spectroscopic characterization of N_2OR from *Hyphomicrobium denitrificans* A3151 (HdN_2OR), which is a methylotrophic denitrifying bacterium that uses methanol as a carbon source. The spectroscopic properties and enzymatic activity of the two forms of HdN_2OR isolated under aerobic conditions are compared to those of other N_2ORs previously reported. From this bacterium, an unusual nitrite reductase containing two type 1 Cu ions and one type 2 Cu ion has already been isolated (*29*, *30*).

MATERIALS AND METHODS

Chemicals—Organic chemicals were obtained from Nacalai Tesque, Sigma, and Aldrich Cos. being of the highest purity available.

Bacterial Growth Conditions—Hyphomicrobium denitrificans A3151 was cultured at 30°C on a defined mineral medium, in which 1% (v/v) methanol served as the primary carbon source and 0.5% (w/v) potassium nitrate as the primary nitrogen source. The medium contain several mineral salts as previously described (*31*). Cells were grown in the medium at 30°C for 3 days under static conditions. The cells (typically 90 g wet weight/50 liters of medium) were washed with physiological saline and been stored at –80°C until use.

Enzyme Assays—N_2OR activity with reduced benzyl viologen as the electron donor was spectrophotometrically assayed by monitoring the oxidation of the chemical electron donor (*32*, *33*). The reaction mixture (3 ml) comprising 50 mM Tris-HCl buffer (pH 7–9.5) containing benzyl viologen (0.40 mM) and sodium dithionite (0.48 mM) was anaerobically prepared in a stoppered 3.5 ml cuvette (light-path length, 10 mm). The enzyme solution (final concentration, 0.1–0.5 μM) was injected into the reaction mixture and then the cuvette was allowed to stand at 25.0°C for 90 min. The enzyme reaction was initiated by the injection of a saturated nitrous oxide solution (final concentration, 1.5 mM). The oxidation of reduced benzyl viologen was monitored as the decrease in absorbance at 550 nm with a UV-2450 spectrophotometer (Shimadzu). The specific activity of HdN_2OR is defined as the amount (μmol) of N_2O reduced per minute per mg protein.

Protein concentrations were determined with a Protein Assay Solution (Nacalai Tesque), with BSA as the calibration standard.

Physical Measurements—Electronic absorption and CD spectra were measured at room temperature with a UV-2450 spectrophotometer and a J-500A spectropolarimeter (JASCO), respectively. An EPR spectrum was recorded with a JES-FE1X X-band spectrometer (JEOL) at 77 K. The copper content was determined with an SP1700VTR inductively coupled argon plasma atomic emission detector (Seiko Instrument).

Preparation of a Crude Extract—Thawed cells were suspended in an equal volume of 50 mM Tris-HCl buffer (pH 7.5) and then sonicated at 180 W for 30 min. Cellular debris was removed by centrifugation at 30,000 ×*g* for 40 min at 4°C.

SDS–Polyacrylamide Gel Electrophoresis—The relative molecular mass of a HdN_2OR monomer was determined and the purity of samples was assessed by SDS-PAGE. The molecular weight standard marker used comprised Low Range SDS-PAGE Molecular Weight Standards (Bio-Rad/U.S.).

Native Molecular Weight Determination—The relative molecular mass of HdN_2OR was determined by TSK-gel $G3000W_{XL}$ (6.0 × 400 mm; Tosoh) gel filtration chromatography. The standard MW-Marker (Oriental Yeast) contained adenylate kinase (M_r = 32,000), enolase (M_r = 32,000), lactase (M_r = 67,000), dehydrogenase (M_r = 142,000), and glutamate dehydrogenase (M_r = 290,000). A sample was eluted with 20 mM Tris-HCl buffer (pH 7.5) containing 100 mM KCl (0.5 ml/min), the absorbance being monitored at 280 nm.

RESULTS AND DISCUSSION

N_2OR Purification and Enzyme Activity—Ammonium sulfate was added to the cell extract to give a concentration of 20% and the resulting precipitate was removed by centrifugation at 10,000 ×*g* for 1 h. The concentration of ammonium sulfate in the supernatant was increased to 80% saturation and the solution including the resulting precipitate was centrifuged. The precipitate containing N_2OR was dissolved in a small amount of water and the resulting solution was dialyzed against 20 mM Tris-HCl buffer (pH 7.5). The dialyzed solution was applied to a DEAE-Sephadex A-50 column (2.5 × 30 cm) equilibrated with the same buffer. After washing the resin with 20 mM Tris-HCl buffer (pH 7.5), the column was eluted at the flow rate of 2 ml/min with a 400-ml linear gradient of 20–400 mM Tris-HCl buffer (pH 7.5). The fractions showing the enzyme activity were eluted with the 200–300 mM buffer, as shown in Fig. 1. The eluate was pooled and the solution was equilibrated with 20 mM Tris-HCl (pH 7.5) containing 100 mM KCl, and then applied to a column (2.5 × 120 cm) of Sephacryl S-200. Three separate colored bands (greenish blue, violet, and orange) were observed. The greenish blue and orange bands included Cu-containing nitrite reductase and methanol dehydrogenase, respectively. The violet band showing enzyme activity was further purified on a Resource Q column (1.6

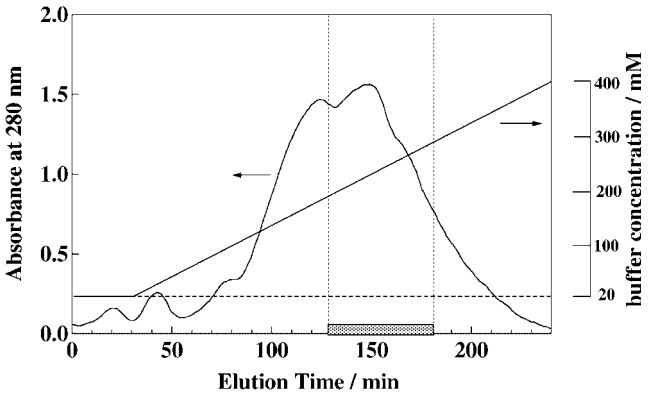

Fig. 1. **DEAE-Sephadex chromatography of HdN_2OR.** The chromatographic conditions are given under Results and Discussion. The fractions were monitored at 280 nm. The bar indicates the pooled N_2OR-active fractions.

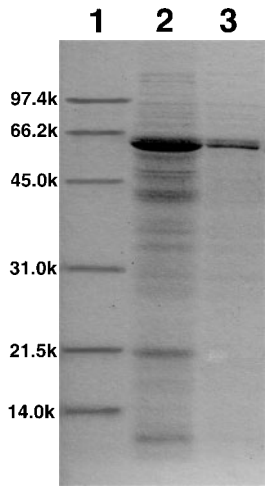

Fig. 3. **12.5%–SDS-PAGE of HdN_2OR.** Lane 1, standard proteins; lane 2, sample treated on a DEAE-Sephadex column; lane 3, final sample (form A) treated on a Resource Q column.

× 3 cm) pre-equilibrated with 20 mM Tris-HCl buffer (pH 7.5), elution being performed with a 0–240 mM KCl gradient (100 ml) at the flow rate of 1.0 ml/min. As shown in Fig. 2, HdN_2OR was obtained as two peak fractions showing activity in the region of 80–120 mM KCl. The purification by HPLC on a Resource Q column was repeated for the second and third peak fractions (violet form A and blue form B in Fig. 2). The monomeric molecular weights of forms A and B were determined by 12.5%–SDS-PAGE. Both forms are homogeneous and have a monomeric molecular mass of 65 kDa (Fig. 3), which is in good agreement with those of N_2ORs of other origins (*1*). Therefore, the molecular weight of HdN_2OR was determined to be 105 ± 30 kDa for both the forms by gel filtration chromatography, suggesting that the enzyme is a homodimer. The Cu contents of forms A and B were estimated to be 9.0 ± 0.2 and 8.2 ± 0.4 atoms per dimer, respectively, although the Cu contents of the N_2ORs from *Pseudomonas nautica* and *Pseudomonas stutzeri* were recently reported to be 10.7 (*14*) and 11.8 (*34*) atoms per dimmer, respectively. These findings might be due to overestimation of the protein concentration using a CBB solution (*35*). Since the catalytic activity is almost the same as that of the N_2ORs (*9*, *14*), HdN_2OR should indeed contain 12 Cu atoms per dimer. No other metals were found in the protein.

When reduced benzyl viologen was used as the electron donor, the pH profiles of N_2OR activity of forms A and B were similar to each other at pH 7–9.5 (Fig. 4). The pH optima of the enzyme activity were pH 8.8, and the specific activity was determined to be 45 ± 5 and 29 ± 3 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ for forms A and B, respectively. The optimal pH values of the specific activity of N_2ORs of other origins have been reported to be in the range of pH 9–10 (*3*, *11*, *33*). The specific activity of violet form A is a little higher than that of blue form B (reduced form), which is very similar to that of the reduced N_2ORs from *Pseudomonas nautica* (*14*) and *Rhodobacter sphaeroides* (*33*). However, the reduced forms from *Pseudomonas stutzeri* (*3*) and *Paracoccus denitrificans* (*6*) have hardly any

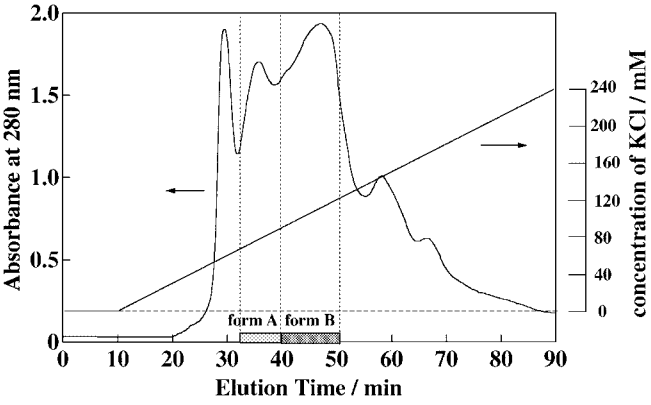

Fig. 2. **Resource Q chromatography of HdN_2OR.** The absorbance was monitored at 280 nm. The bars designated as forms A and B indicate the two portions of the pooled N_2OR fractions.

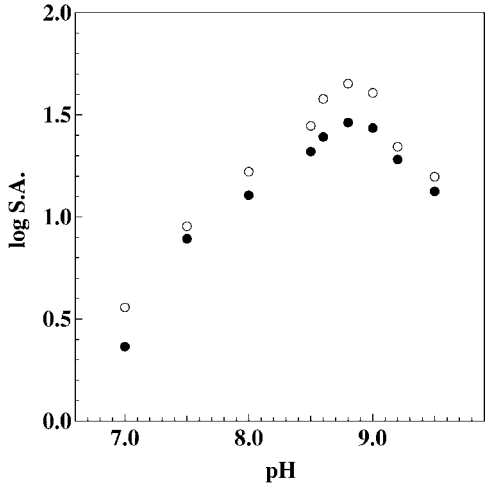

Fig. 4. **pH dependence of the enzyme activity of form A (opened circles) and form B (closed circles) in 50 mM Tris-HCl buffer (pH 7–9.5) at 25.0°C.** The ordinate shows the common logarithm of specific activity (see "MATERIALS AND METHODS").

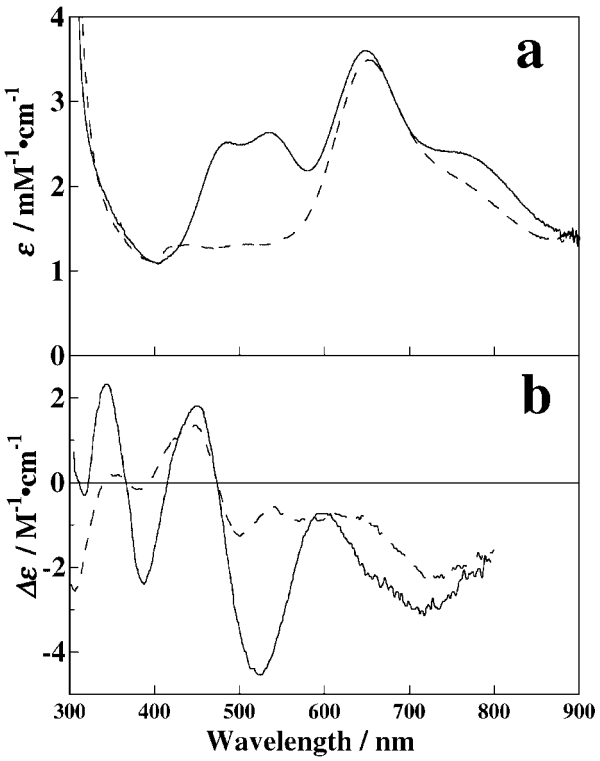

Fig. 5. **Visible electronic absorption (a) and CD (b) spectra of form A (solid lines) and form B (broken lines) in 20 mM Tris-HCl buffer (pH 7.5) at 25.0°C.**

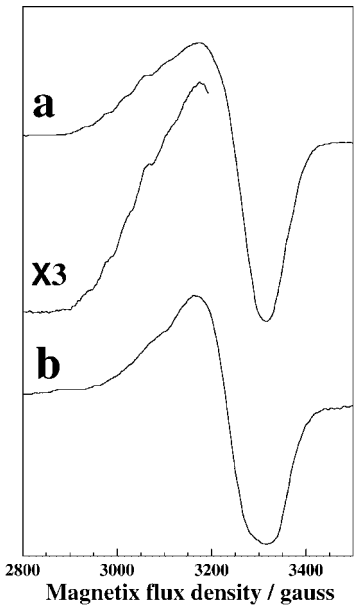

Fig. 6. **X-band EPR spectra of form A (a) and form B (b) in 20 mM Tris-HCl (pH 7.5) at 77 K.**

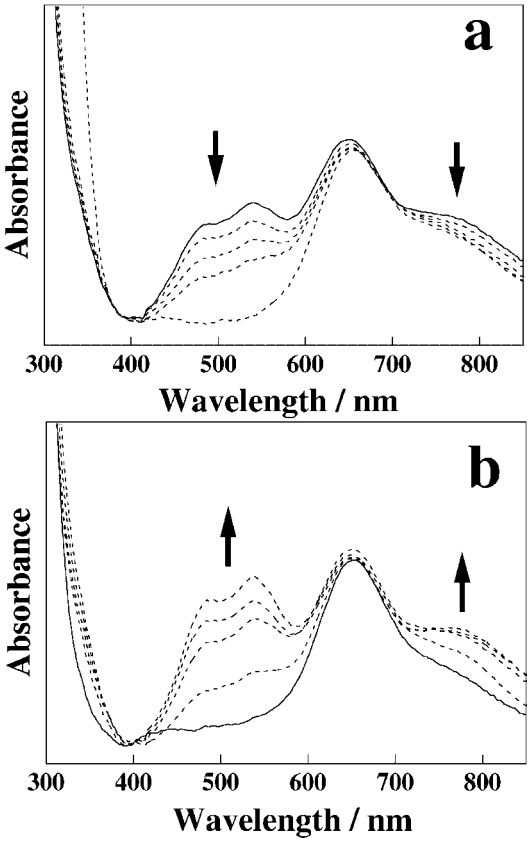

Fig. 7. **Absorption spectral changes of form A and form B on titration with redox reagents in 20 mM Tris-HCl (pH 7.5) at 25°C.** (a) Absorption spectra of form A with the addition of 0.25, 0.5, 0.75, and 1.0 equivalents of sodium dithionite under anaerobic conditions. (b) Absorption spectra of form B with the addition of 0.25, 0.5, 0.75, and 1.0 equivalents of potassium ferricyanide under aerobic conditions.

activity. An aerobic preparation of N_2OR usually gives a low activity "pink" form (*28*). However, the aerobic form (form A) of HdN_2OR exhibits high enzyme activity since the enzyme gives no "pink" form, which is a unique character of HdNIR.

Visible Electronic Absorption, CD, and EPR Spectra of HdN_2OR—In Fig. 5a, the visible absorption spectrum of form A is characterized by significant absorption peaks at 480 nm (ε = 2,500), 540 nm (ε = 2,600), and 650 nm (ε = 3,600 $M^{-1}\cdot cm^{-1}$), with a shoulder around 780 nm. This spectrum is a little different from those of known "purple" and "pink" forms of N_2OR (*25–28*). It is generally recognized that the intense absorption bands at 480 and 540 nm with a shoulder at 780 nm arise from A_{mv}, and the 650-nm band is assigned to Z_{mv}^* (*25–28*). A characteristic intense peak at 560 nm for Z_{ox} was not observed in the visible absorption spectrum of form A. Although the "purple" and "pink" forms of most N_2ORs have been known to contain both Z_{ox} and Z_{mv}^* with A_{mv} (*28*), form A under aerobic conditions would have only Z_{mv}^* with A_{mv}. The CD spectrum of form A exhibits two positive peaks at 340 and 450 nm, and three negative peaks at 380, 520, and 720 nm (Fig. 5b). In Fig. 6a, the 77-K EPR spectrum of form A shows an axial signal with $g_{//} = 2.18$ and $g_{\perp} = 2.06$, and the hyperfine structure shows seven equidistant lines ($A_{//} = 4.5$ mT) characteristic of A_{mv}.

As shown in Fig. 5a, the visible absorption spectrum of form B aerobically isolated is characterized by an intense absorption band at 650 nm (ε = 3,500 $M^{-1}\cdot cm^{-1}$), which is assigned to Z_{mv}^*. The CD spectrum of form B exhibits two

positive peaks at 360 and 450 nm, and four negative peaks at 380, 500, 580, and 720 nm. Form B gives a broad featureless EPR signal with g = 2.06 (Fig. 6b). The visible, CD, and EPR spectra of form B are very similar to the corresponding spectral data of "blue" N_2OR containing A_{red} and Z_{mv}^* (*25–28*). Interestingly, form B is stable under aerobic conditions, although "blue" N_2ORs of other origins were only obtained on reduction of the corresponding "purple" and "pink" forms under anaerobic conditions. The redox potential of Cu_A in HdN_2OR would be positively shifted compared with those of other N_2ORs because Cu_A in form B is reduced (colorless A_{red}) and only Z_{mv}^* is observed as a blue chromophore.

When form A was reduced with sodium dithionite under anaerobic conditions, the absorption spectrum gradually changed with increasing dithionite, as shown in Fig. 7a. Decreases in the three absorption peaks at 480, 540, and 780 nm were observed, and the final spectrum was very similar to that of form B. The reduced form A obtained on the addition of one equivalent dithionite was stable under aerobic conditions for weeks at 4°C as the case of form B. The difference absorption spectrum, between form A and dithionite-reduced N_2OR, is analogous to the absorption spectrum of an N_2OR mutant containing only Cu_A (*36*). When potassium ferricyanide was added to form A, no spectral change was observed. The visible spectrum of form B is characterized by the intense maximum at 650 nm. On oxidation of form B with one equivalent of potassium ferricyanide, a visible spectrum similar to that of form A was observed under aerobic conditions (Fig. 7b). The oxidized form B was purified by Resource Q chromatography under the same conditions, showing exactly the same retention time as that of form A. When sodium dithionite was added to form B, no spectral change occurred. Accordingly, the redox reactions between forms A and B are reversible. Forms A and B are not isozymes, but are essentially the same protein. However, they exhibit different chromatographic behavior in the oxidation state. Form A is in an oxidation state containing A_{mv} and Z_{mv}^*, and form B is in a reduction state containing A_{red} and Z_{mv}^*; therefore the redox site of HdN_2OR which brings about the reversible spectral change is Cu_A.

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