CHEMISTRY LETTERS, pp. 1053-1056, 1985.

ISOLATION AND SOME PROPERTIES OF A NOVEL VIOLET COPPER PROTEIN FROM A DENITRIFYING BACTERIUM, ALCALIGENES SP.

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A novel violet copper protein which is a candidate for N_2O-reductase, was purified from a denitrifying bacterium, *Alcaligenes* sp. NCIB 11015. Molecular weight is 120000. Copper content is 0.261%. The absorption, CD, EPR, and EXAFS studies have been carried out for this protein.

Denitrification is an anaerobic respiration process with distinct terminal oxidoreductases; nitrate, nitrite, nitric oxide, and nitrous oxide reductases. In the last step, $N_2O \longrightarrow N_2$, copper atom is an essential element.[1,2] A violet-pink copper protein with the molecular weight of 120000 was purified as nitrous oxide reductase in *Pseudomonas perfectomarinus*.[3,4] Snyder and Hollocher [5] reported that the chromatographic behavior of this violet-pink protein was different from that of the activity of N_2O reduction. However, Zumft et al.[6] found that the difference of the chromatographic behaviors resulted from the effect of oxygen and confirmed that the violet-pink copper protein is N_2O-reductase.

We purified a violet copper protein as a candidate for N_2O-reductase from a denitrifying bacterium, *Alcaligenes* sp. In this paper, we report some spectral properties of this novel copper protein.

Alcaligenes sp. NCIB 11015 was grown anaerobically with nitrate as an electron acceptor in the broth medium reported previously.[7] About 200 g wet weight cells were harvested from 100 l medium by a continuous-flow centrifuge. Cells resuspended in 0.1 M (1 M=1 mol dm^{-3}) K-phosphate buffer, pH 7.0, were disrupted by sonic vibration at 9 kHz, 200 W (Kubota Model 200 M) at 0 °C. The homogenate was spun for 90 min at 205700xg, and the resulting supernatant was fractionated with $(NH_4)_2SO_4$ between 40-90% saturation. The following purification procedures were basically the same as reported in *Ps. perfectomarinus*[4]; chromatographies on DEAE-cellulose and DEAE-Sepharose (Whatman and Pharmacia, 2.5 cmx 45 cm; 10 mM Tris-HCl, pH 7.5), and gel filtrations on Sephacryl S-200 and S-300 (Pharmacia, 2.5 cmx45 cm) were carried out to purify the violet copper protein. Instead of the preparative isoelectric focusing, the chromatofocusing was used (the protein was focused at around pH 6.2); a Polybuffer exchangers PBE 94 column (1 cmx38 cm) was equilibrated with 25 mM imidazole-HCl, pH 7.4, and the eluent was polybuffer 74-HCl, pH 5.0. Materials for chromatofocusing were purchased from Pharmacia. The purified violet copper protein was homogeneous in

the chromatographic and the polyacrylamide gel electrophoretic criteria.

The molecular weight of the violet copper protein was determined as 120000 by gel filtrations on Sephacryl S-200 and S-300 at pH 6.2 and 7.5. Copper content of the purified protein was determined by atomic absorption spectroscopy, and it was 0.261% Cu on the basis of the dry weight of the protein.

Figure 1 shows the absorption spectra in 10 mM K-phosphate buffer, pH 6.2. There were no changes on the absorption spectra at pH 6.2, 7.0, and 7.5. The spectrum has a maximum at 280 nm and shoulders at 255, 260, 265, and 290 nm. In the visible region, oxidized form of the protein has two peaks at 545 and 780 nm with shoulders at 360, 480, and 650 nm. Molar extinction coefficients were 2870, 3800, 6480, 4220, and 2600 $M^{-1}cm^{-1}$ at 780, 650, 545, 480, and 360 nm, respectively, on the basis of the dry weight and the molecular weight of 120000 of the protein. The ratio of A_{280nm}/A_{545nm} was 18 for the most purified sample. The absorption at 545 nm in the violet copper protein is higher than those in the similar type copper proteins reported previously.[3,4,8] The protein which was reduced by the addition of dithionite had a maximum at 650 nm with shoulders at around 550 and 750 nm (Fig. 1). The color of the reduced sample showed faint blue. The reduced form was reoxidized by the addition of ferricyanide. Very slow autoxidation was observed in the dithionite-reduced sample during gel-filtration to remove dithionite. When the protein of the violet form at pH 7.5 (10 mM Tris-HCl) was frozen at -20 °C for a few days, the color turned blue, and the spectrum of the thawed solution showed the similar pattern to the dithionite-reduced form. This effect of freezing was also found in the copper protein from *Ps. perfectomarinus*.[4] The freezing effect was not strong at pH 6.2 (10 mM phosphate buffer). Copper content did not reasonably decrease in the frozen and thawed violet copper protein.

By the addition of cyanide at the final concentration of 0.5 mM, absorption peaks of the oxidized violet copper protein disappeared in the visible region at pH 6.2. After the cyanide treatment, the solution was dialyzed against the same buffer, and the copper content of the sample decreased to 0.106%.

The circular dichroism (CD) spectrum is shown in Fig. 2. It exhibited two positive peaks at

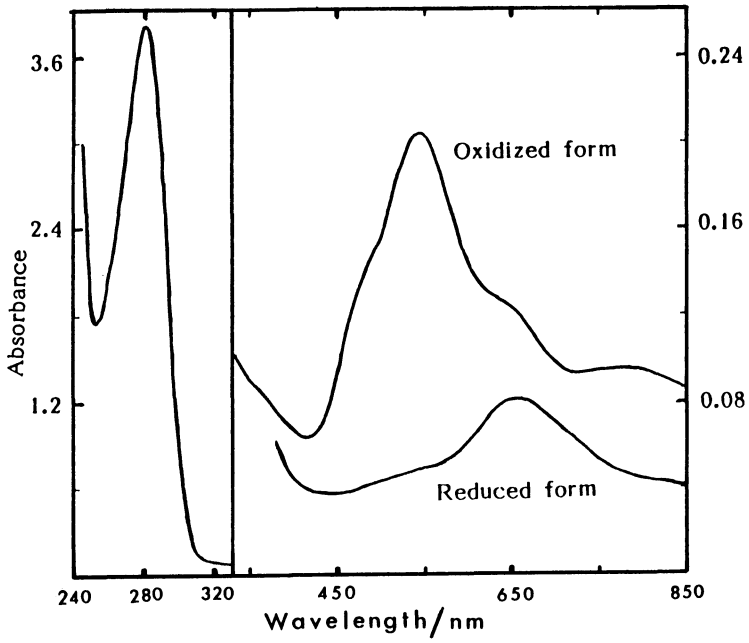

Fig. 1. Absorption spectra of the oxidized and reduced forms of the violet copper protein in *Alcaligenes* sp. (10 mM K-phosphate buffer pH 6.2, protein concentration: 3.7 mg/ml, light path: 1 cm)

347 and 458 nm with a shoulder at around 420 nm, and four negative peaks at 318, 382, 522, and 670 nm with a shoulder at around 740 nm.

The X-band EPR spectrum of the frozen violet copper protein is illustrated in Fig. 3. The spectrum was able to be interpreted as axial with spin Hamiltonian parameters $g_{//}=2.24$, $g_{\perp}=2.07$, and $A_{//}=0.0167\ cm^{-1}$ (160 G).

In order to investigate the copper site, the EXAFS spectra have been examined. The technique of EXAFS has proven to be a powerful method for investigating the structure around a specific metal even in non-crystalline materials. The X-ray absorption measurement was carried out on the storage ring of the Photon Factory at National Laboratory of High-energy Physics (2.5 GeV, 70-120 mA). Purified copper protein of violet form was freeze-dried and used for the EXAFS. Spectra were registered in a transmission mode at room temperature. The EXAFS spectra were analyzed by the standard procedures. Figure 4 shows

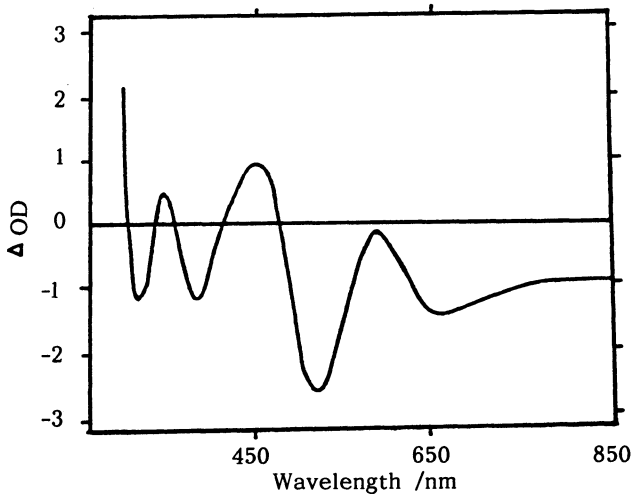

Fig. 2. CD spectra of the oxidized form of the violet copper protein (10 mM K-phosphate buffer pH 6.2, protein concentration: 158 mg/ml, light path: 1 mm).

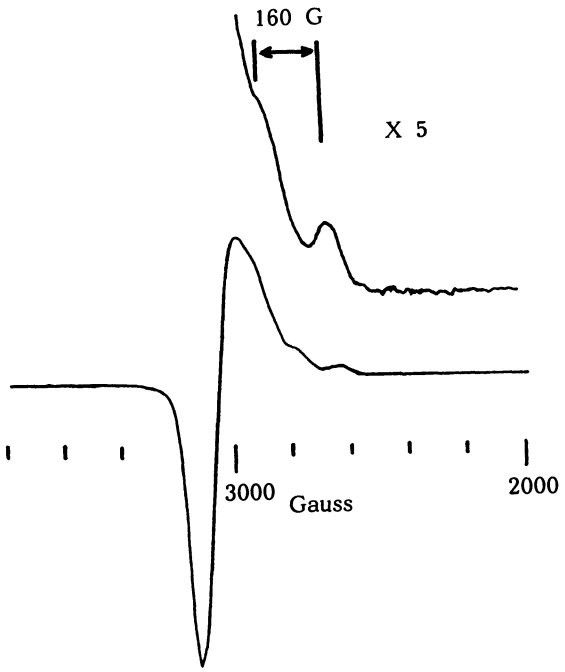

Fig. 3. EPR spectra of the oxidized form of the violet copper protein at 150 K.

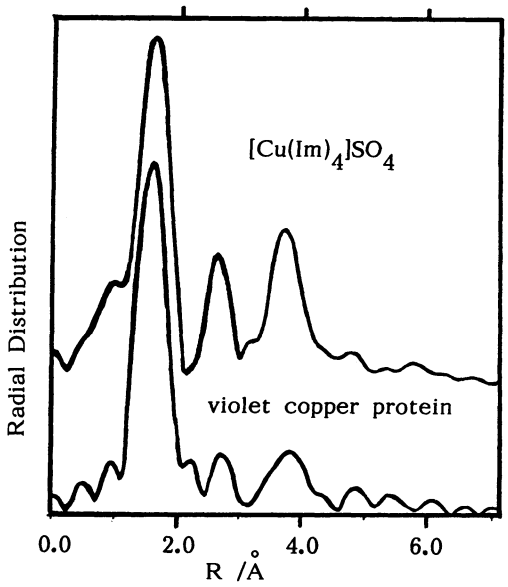

Fig. 4. Fourier-transform of the EXAFS spectra for the violet copper protein and $[Cu(Im)_4]SO_4$ (not corrected for the phase-shift).

the radial distribution from Cu atom with that of the reference compound of $[Cu(Im)_4]SO_4$ (Im=imidazole). The distribution function of the protein shows a main peak centered at about 1.8 Å and two satellite peaks at 2.9 and 3.8 Å. The two satellite peaks are characteristic for the interaction between copper and imidazole. This suggested the existence of imidazole of histidine around a copper atom, though the coordination number was not determined.

Many copper proteins are known till the present time. Those copper proteins are classified into three types by their spectroscopic and magnetic properties. The 'blue' copper proteins (type I) show strong absorption at around 600 nm. The proteins of type II, such as amine oxidase and galactose oxidase, show only very weak absorption bands in visible region. The type III copper proteins show diamagnetic owing to the pair of two copper atoms. Though the violet copper protein shows the type II copper EPR signal, the absorption spectrum is a novel one.

The copper atom is known to be an essential element in the step of $N_2O \longrightarrow N_2$.[1,2] The violet-pink copper protein of *Ps. perfectomarinus*, which was identified as N_2O-reductase,[4,6] shows similar absorption specra to the violet copper protein of *Alcaligenes* sp. The violet copper protein reported here is thus the most probable candidate for N_2O-reductase. Since there are some complicated problems to obtain consistently active enzyme preparation *in vitro*,[6] we can not yet identify this violet protein as N_2O-reductase in *Alcaligenes* sp. The function of the violet copper protein will be progressed in the light of information from physico-chemical studies.

We thank the staff of the Photon Factory at National Laboratory of High-energy Physics for their providing beam time and for their kindly giving us useful advice and asistance.

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(Received April 27, 1985)